CRYPTOBIOTIC STAGES IN BIOLOGICAL SYSTEMS

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# PREFACE

Specialization in modern biology has assumed an extreme form. In this situation, even within a small country such as Israel, communication between specialists could disappear entirely if it were not actively encouraged. At our annual 'Oholo' conference, procedures which foster the desired interdisciplinary communication in biology are being followed. The participants at these conferences represent widely divergent approaches to the biological method. They come from the different scientific institutions of Israel, and increasingly also from distant countries now that funds and facilities for travel have become more easily available. Subjects upon which discussion at these meetings has been centered* belong to areas in which different biological disciplines overlap and interlock. Ample allowances of time for formal and informal discussions, conducted against the serene background of an ancient and beautiful lake in Galilee, have complemented and greatly enhanced the value of the formal programs.

The titles of the annual conferences convened as from the spring of 1956 at Oholo have designated a very wide area of discussion; yet this is, in fact, narrower than the one which we have covered.

The subject of this year's meeting, 'Cryptobiotic Stages in Biological Systems', was one which has received less attention in the scientific literature than it probably deserves. With the notable exception of an important recent review on 'The Problem of Anabiosis or Latent Life: History and Current Concept', by Prof. D. Keilin, the interdisciplinary approach in this area has been the exception rather than the rule. We are hoping that this book will help in some ways to answer the need.

* 1956 Bacterial Genetics
1957 Tissue Cultures in Virological Research
1958 Inborn and Acquired Resistance to Infection in Animals
1959 Experimental Approach to Mental Diseases

The scientific proceedings of the 'Oholo' conference are now being published for the first time in book form. The Editors gladly take this first opportunity to express their thanks to the participants in these conferences and to the organizing committees for their generous gifts of time and effort which made this publication possible.

The Board of Editors hereby gratefully acknowledges the help of Mrs. B. Wolman in the preparation of the monograph and of Mr. L. Hirschberg-Bar-Sinai in the editing of the Round Table discussion.

# CRYPTOBIOTIC STAGES IN BIOLOGY

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The term cryptobiosis was first introduced by Keilin[1] in his Leeuwenhoek Lecture given at the University of Cambridge. He intended this term to include all forms of latent life, such as the anabiosis suggested by Preyer[2] and the abiosis introduced by Schmidt[3]. In his discussion of this pseudo-lifelessness, he treated the dormant state as though it were synonymous with cryptobiosis: but in his classification of the related terms he differentiated between them regarding the dormant state as encompassing hibernation, diapause, and quiescence, states where there is no growth but some metabolic activity. It would appear to me that, in many areas of biology, the term dormancy has been used to denote latent life, a state that shows metabolic activity only when a suitable environment arises. I can agree with Keilin that hibernation is not the same as cryptobiosis, in that in the former there is a positive metabolism even though it may be reduced to a low level, whereas in the latter no positive metabolism is perceptible. In this discussion, however, I shall use the term dormancy as it has been used in the past—by bacteriologists to describe bacterial spores and by zoologists to describe protozoan cysts. In these cases it is really analogous to cryptobiosis. As indicated in Keilin's outline, among various forms that exhibit reduced metabolism to a marked degree, there is no sharp dividing line between a state of 'hypometabolism' and 'ametabolism'. In dealing with the state of cryptobiosis, therefore, one must necessarily refer to some of the borderline cases.

A state of cryptobiosis is certainly not limited to any single area of biology. One can find good examples among plants, animals and micro-organisms. Among these, one needs to mention: the seeds of plants; plant buds; the dehydrated forms of all kinds of micro-organisms, such as protozoa; bacteria,

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fungi, and algae; viable forms rendered inactive by freezing; the cysts of protoza; and the spores of bacteria. I wish to discuss each of these in some detail under the headings of dehydrated forms, seeds, buds, cysts, insects, and spores.

## DEHYDRATED FORMS

Keilin refers to this as a state of anhydrobiosis. In this state, forms seem to be lifeless because of lack of water. The inert, dry, yet viable protozoa were first observed by Leeuwenhoek when he showed that gutter-sand and dirt which had been kept in his laboratory for some time in a very dry condition, contained viable protozoa. They could be observed to be living and active immediately after sterile water was added to the material. Similar observations have been made by biologists from that day to the present. It is now well established, therefore, that many protozoa and some metazoans such as rotifers, tardigrades, and nematodes, can be dried to the point where there is virtually no moisture left; these can be shown to be viable and unharmed, however, when rehydrated. Not only will they withstand this desiccation, but they can also be stored for long periods of time in the desiccated condition without, seemingly, much loss of viability. Similar experiments have been performed with many forms of fungi and bacteria. I am not familiar with the literature on algae, but it certainly must be true that many of their forms also can be desiccated in like manner and remain viable. This seems to be nature's way of preserving these forms through periods of drought and transport.

It cannot be presumed, however, that complete desiccation and storage in the dry state does not damage the cells in some manner. This has been shown with bacteria that have been preserved by desiccation or lyophilization[4, 5]. When one makes quantitative studies of the number of viable cells present before and after drying and rehydration, one can show that many of the individual cells die in the process. Why some cells live and others die is certainly not known. It can be due to a

difference in the biological state of the individual cells. It is also possible that the cells that die secrete substances which help to preserve and protect those that remain. It has been shown both with bacteria and protozoa that a few cycles of repeated drying and rehydration will kill all the cells if no opportunity is given for them to grow and repair damage between cycles. It is also clear from experiments that have been performed on bacteria, that damage can occur both during drying and rehydration as well as during the period of storage in the dry state[6]. This is clear from the varied quantitative results obtained with different methods of drying and rehydration. The degree of killing during drying is influenced greatly by the composition of the medium in which the cells are dried. An abundance of colloids will generally help to protect the cells; whereas metabolic end-products, such as acids and other small organic compounds will have an opposite effect, particularly as the concentrations are increased with the removal of water. The protective action of colloids, such as proteins, starches, and dextrins, may be due in part to a counteraction of the toxic effect of accumulated metabolic byproducts or to an antioxidant effect. The temperature of the material during drying can also have a marked effect. In general, the lower the temperature is during drying, the less the damage. It is for this reason in part, perhaps, that many cells can be preserved best when they are dried from the frozen state. Here, however, one does need to consider the additional damage that may occur during freezing. If cells are dried from the frozen state, it should be remembered that the rate of freezing is important as well as the nature of the menstruum that is frozen.

Damage is also incurred during rehydration. With dehydrated bacteria and yeasts, the temperature and the rate of rehydration are important factors in controlling the extent of damage. Desiccated cells may sustain injury to their cell walls, so that during rehydration, soluble constituents leach out[7]. If the temperature and other environmental conditions are such that growth can be initiated at the outset, it may be possible for the

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cells to repair this damage before the leaching and other subsequent undesirable changes become lethal. The manufacturers of bakers' yeast have been able to dry yeasts and retain a high degree of viability by carefully controlling the strain, growth conditions, method of desiccation, and method of rehydration. The problem is complex, and it is likely that optimum conditions will vary with each variety of cell.

In bacterial cells, there seems to be some correlation between the size of the cells and the ease with which they can be preserved by desiccation. In general, the smaller cells can be preserved in this manner more successfully than the larger ones. It is easier, therefore, to keep cocci alive in a dry state than yeasts, and the vegetative cells of the Gram positive spore-forming bacteria are more easily damaged by drying than the smaller Gram positive lactic acid bacteria[8].

With most dried cells there is a slow 'die off' during storage. Here again, protective additives can have a marked effect on the stability of the cells. It is felt by many that the damage during storage may be due in part at least to oxidation and, therefore, that cells will be more stable if stored in an inert gas than in air or oxygen. This has been found to be true particularly with the viruses. Here, storage in the presence of air is quite damaging[9]. With these, to attain long time livability, it is necessary to dry and store the cells in an inert atmosphere at all times. Exposure to air even for a short period may be quite injurious, because, if the air comes in contact with the cells, enough oxygen is absorbed to produce products that are lethal. This lethal effect may be due to the formation of peroxides or hydroperoxides, which are known to be very toxic.

Cells of protozoa, fungi, and bacteria that are properly dried can be kept viable for long periods of time. By this, we do not mean that all the cells in the population remain alive, but enough do, to allow one to recover an active culture when the material is rehydrated. This, of course, can happen even though only a very small fraction of the initial population remains viable. In our own laboratories, we have recovered active

cultures of pathogenic streptococci from lyophilized preparations that have been stored under an inert gas at room temperature for nineteen years.

## SEEDS

It is by means of dormant seeds that nature provides a way of continuing the existence of many plant species. There are two types of these[10]. One, a seed that remains dormant only as long as it remains dry. The process of germination is set in motion as soon as the seed comes in contact with moisture. The other type is a seed that remains dormant even though it has absorbed all the moisture that is needed. In this case, germination does not proceed until some external trigger mechanism sets the process in motion.

The first represents a type of dehydrobiosis common to the seeds of cultivated plants, such as the grains and maize. These seeds remain dormant during storage because they are kept dry. There may be a short period of after-ripening between the time they are harvested and the time they are planted, but in these seeds this period is short and occurs during the normal period of storage. When such seeds are planted, they imbibe moisture rapidly and germinate in a few days. The wild parents from which these cultivated plants have been derived probably did not have seeds of this kind, but through a long period of cultivation and natural selection, types have been developed which produce seeds that do not require any special trigger mechanism for germination. This is desirable in order that all the planted seeds germinate at the same time to produce plants that mature at about the same time. With seeds of this kind, there is some difference of opinion as to whether or not they are in a real state of cryptobiosis. In many studies on such seeds, it would appear that a slow rate of respiration goes on during the storage period even though the moisture content is low, and that this rate of respiration increases as the moisture content is increased[11]. It is not certain, however, whether this respiratory

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activity is from the seeds themselves or from a slow metabolism carried on by fungi, found on the seeds, which increase their rate of respiration when the moisture content is raised above the very minimum levels[12]. Certainly when such seeds are kept in environments where the moisture content is maintained below the critical levels of from 6—10% (depending upon the type and species), there is very little, if any, metabolism going on within the embryo of the seed itself. If this is not a true cryptobiotic state, it certainly is not far removed from one.

The seeds of many weeds, flowering plants, vegetable plants, fruits, and legumes usually require special trigger mechanisms to germinate, in addition to the imbibition of water. In these, there may be a period of after-ripening or other special requirements necessary. Some of these special requirements are the removal of inhibitors, the absorbtion of inducers, the breaking or damaging of hard seed coats, and the need for exposure to light to permit photoinduction. Whatever the mechanism—and it is often different for different species of seeds—many may remain dormant for many months or even years in an environment where one would normally expect them to germinate[11]. Those of you who have lived in the northern half of the United States of America, or in other areas that have similar climate, will know what I mean when I remind you of the difficulty of getting rid of crab grass in your lawn. Year after year one can remove new shoots in order to prevent the formation of any new seeds, and yet have the weed continue to come up year after year. Here a photoactivation is undoubtedly involved, but the biochemical mechanism that is triggered by this means is certainly not understood. In other cases, contact with oxygen through an exposure to normal atmosphere is necessary. Thus, many farmers have experienced the phenomenon of seeing new types of weeds appear in fields following deep plowing, weeds that had not been seen in those fields for many years. Here the deep plowing presumably brought deeply buried seeds to the surface where they could become exposed to oxygen and perhaps light, or both.

In many seeds there may be inhibitors of germination found in the fruit that covers the seed, or in the seed coating[13]. As long as these are present, no germination can take place. During storage in the soil or other suitable environment, these inhibitors may leach out slowly or may even be destroyed by microorganisms. Following their removal, germination can take place in a normal manner. In a single batch of seeds, not all of them will free themselves from the inhibitors at the same time. The different individuals may germinate at various times over a long period, thus helping to insure the propagation of the species. Germination in some seeds is triggered by a periodic change of temperature, that is, from storage at a low temperature to storage at a high or moderate one. In some cases, more than one cycle of temperature change may be necessary. The mechanism involved here, as it must be in many of the other cases, must be biochemical, a temperature change being necessary for the proper sequence of biochemical changes. Unfortunately, these phenomena are not understood, so that we do not have even fragmentary knowledge of the mechanism.

Some seeds, like those of the legumes, have a very hard coating that is practically impervious to moisture and oxygen, so that the seed embryo within the shell is kept in a state of anhydrobiosis, even though the environment outside the shell is conductive to germination[14]. These seed shells have interesting properties in that they will allow the moisture within the seed to escape, preserving the embryo through dehydration, but will not let water pass in the reverse direction. Such seeds can be made to germinate quickly by mechanically or chemically injuring the shell in order to let water in. In nature, such seeds may depend upon the action of bacteria from the outside to damage the coat, so that moisture can reach the embryo.

## BUDS

The buds of plants usually remain dormant for a shorter or longer period, and many never open up. Whether or not they vegetate depends upon a number of conditions: such as favor-

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able temperature, light, humidity: a nutrient supply; and certain conditions of equilibrium within the plant[15]. If a bud remains dormant for more than a year after its formation, it usually becomes latent, in which case it will open and grow only under rather unusual conditions[15].

Under normal conditions, buds will remain dormant only during the winter months. The exposure to the low temperatures of winter appears to be an essential feature in the breaking of the dormancy. When the warmer temperatures occur in the spring, the buds will begin to grow normally. The need for this exposure to the low temperatures of winter has been evidenced in some of the warmer climates where in certain years the temperature does not get low enough to properly condition these buds. Such buds, when they do begin to grow in the spring, are abnormal; and any flowers that are produced, are irregular. The cause of dormancy in buds is not well understood. Some believe it is due to a lowering of the oxygen level, but apparently this cannot be substantiated by the experimental evidence that is available. Considerable research has been directed toward breaking this dormancy by chemical means. For this purpose, ethylene, chlorohydrin, thiocyanates, thiourea, dinitrocresol, and dinitrophenol have been used, but there are many buds which do not respond to these chemicals.

Latent buds may remain in the bark of the tree during the entire life of the plant and, as mentioned earlier, grow only under very unusual conditions. These latent buds are not loose in the bark, but continue to be attached and directly connected with the annual ring from which they originate. When a limb or stem of some size is cut, the numerous shoots that spring from near the cut edges of the bark come from these latent buds. These are sometimes called adventitious buds. Practically nothing is known about the trigger mechanisms that initiate growth in these, but certainly this is nature's way of providing a mechanism for growth of the plant if the normal buds are destroyed. The most interesting examples of this that I have seen, are shoots that develop from these adventitious buds on

the burls of redwood trees when these are placed in a dish with moisture.

The trigger mechanism involved here must certainly be biochemical, and an understanding of this would not only be very interesting but also very useful.

## CYSTS

Among the protozoa, the cysts are perhaps the best example of a cryptobiotic state. In many respects these are similar to the seeds of plants and the spores of bacteria in that, while they remain as cysts, there is virtually no metabolism going on. In many cases these cysts remain as such only when they are stored at a low temperature or when they are dehydrated, but in other cases the cysts can remain stable for long periods of time in media where one would normally expect them to undergo excystment. With these, as in the case of some plant seeds, external factors other than moisture are needed to trigger the excystment process[17]. Although a large number of environmental factors have been implicated by various workers, there is so much conflicting evidence that one cannot be certain which factors are important. In a few instances carefully controlled experiments tend to show that the principal factors are catalytic amounts of certain organic compounds, such as the potassium and sodium salts of l-malic, citric, acetic, fumaric, oxalic, and lactic acids, with some carbohydrates acting as co-factors. These acids were even more effective when supplemented by low molecular weight organic compounds isolated from hay infusion. These unknown compounds were effective in concentrations as low as $10^{-8}$. In the absence of these acids and co-factors, the cysts can remain dormant for long periods in a medium that will support the growth of the normal adult and at temperatures that would favor this growth[18]. In some instances these organic substances may be supplied by bacteria that are growing in the medium.

In addition to the cysts, vegetative forms of protozoa of

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various species may be put into a state of cryptobiosis by dehydration, as mentioned earlier, and also by a lowering of the temperature. Thus, many nonencysted forms of protozoa can be preserved for long periods of time by being suspended in a frozen medium. Here, there is a great deal of variation between species, some are sensitive to freezing whereas others are very resistant. Here, also, as with bacteria, the method of freezing, the ultimate temperature, and the constancy of the temperature at which they are stored can have an important bearing upon the longevity of the individual cells.

## INSECTS

The eggs of insects, for example, the eggs of mosquitoes, have many properties in common with plant seeds and the cysts of protozoa. Under proper conditions these can be kept for long periods of time without loss of viability. Eventually they do die, which may indicate there is a low level of metabolism which uses up the reserve food. If stored at room temperatures, the life span is materially shortened.

The hatching of these eggs may be compared to the germination of seeds or the excystment of protozoan cysts. In most cases the hatching process needs to be triggered by some external factor, such as a favorable temperature, along with a lowering of the oxygen tension[19]. In nature, these eggs may thus be preserved through the winter, but when spring comes and the temperature gets high enough for substantial growth of bacteria, the necessary lowering of $O_2$ tension can occur through the metabolic activity of these organisms, and, as soon as the oxygen tension is brought to the proper low level, hatching will take place. In the laboratory the same thing can be accomplished by replacing the oxygen with nitrogen. Thus, persons who are working with mosquito eggs can store them for a long time without much loss of viability and hatch them at will. There are some mosquito eggs, however, that have a more complex trigger mechanism, in that they may require a change in temperature

from a low to a high value before they can be made to hatch[20]. Hence, there are certain species of mosquitoes that may lay their eggs in the spring or summer, but the eggs will not hatch until they have been stored over the winter and then brought to the proper temperature in the spring[21]. To the best of my knowledge, the biochemical changes that are involved are not known.

In addition to the dormancy that is exhibited with these eggs of insects, it is also possible to get dormant forms in almost any of the developmental stages of the insects. These can be preserved in a seemingly dormant state by a proper lowering of temperature. Many insects, therefore, will survive the frozen state. In general, however, there is a limit to how low the temperature can be brought. According to Prosser[22], at the University of Illinois, the temperature can be lowered until about 70% of the body water is frozen; below that, the organism dies. Depending upon the species, the temperature may be brought considerably below the freezing point of water before a lethal temperature is reached. It is presumed that a considerable amount of organic material is dissolved in the remaining water, so as to effectively lower the freezing point. In other words, the organisms have a built-in antifreeze system. At these low temperatures, the metabolism is reduced almost to, if not to, zero, so that these also may be regarded as being in a state of cryptobiosis.

## SPORES

Among the bacteria, the spores are the best examples of a cryptobiotic state, although vegetative cells, as indicated above, can be brought into such a state through drying or freezing.

The spores that are produced by some species of aerobes and anaerobes are the most resistant dormant cells known. Freshly produced and properly separated from their growth medium, they are truly inert and perhaps the best examples of a cryptobiotic state that can be found. Many studies have been made on the respiratory activity of such cells, but the data are conflicting.

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Much of this is due to an improper understanding of the ease with which some of the cells can be made to germinate. Since the germinated cells do show considerable metabolic activity, one needs to exercise extreme care to insure that, in studies on spores, one does not deal with germinated cells[23].

Before continuing this discussion we need to define our terms. The conversion of a spore to a mature vegetative cell involves two steps. The first, and one that can occur rapidly, involves only the activation of dormant enzyme systems that change the dormant cell to one that has an active metabolism. The second step involves the initiation of growth and finally the emergence of the new vegetative cell. Bacteriologists who are engaged in research on spores limit the term germination to the first step only.

Several things occur simultaneously in this first change; the loss of refractility, the loss of heat resistance, and the gain of stainability. Recently we have found that the loss of resistance to octyl alcohol occurs, also. Germinated spores or vegetative cells are very sensitive to this chemical whereas spores are extremely resistant. During the initial change, many enzymes that are dormant in the spore become active and begin to function. This will include practically all the enzymes that are needed for energy for growth, such as those concerned in the electron transport system. Thus, clean spores will not show any oxygen uptake with glucose as a substrate, but germinated spores (using this term in the sense in which it was defined above) show a very rapid uptake of oxygen with that substrate.

A number of agents can be used to trigger this germination process, such as a mixture of amino acids and nucleotides, with or without previous heat shock, depending upon the species and past history of the spores. Some spores can be germinated with single amino acids, others with nucleotides alone, and some with various kinds of chelating agents. Regardless of the trigger mechanism used, dipicolinic acid, calcium ions, and some polypeptides are released from the spore simultaneously with the activation of the enzyme. Most workers believe that the

release of these substances is a necessary prerequisite for the activation of the enzymes. They believe that the enzymes are rendered dormant by incorporation into a complex. This complexing not only makes them inactive but also renders them resistant to heat and chemicals.

In bacterial spores we find a number of phenomena that are common to other dormant systems. Aging simplifies the germination requirements. This is comparable to the after-ripening process that goes on in certain seeds. Many fresh spores require heat shock in addition to the presence of the germination nutrients. Upon aging, the need for heat may disappear. Also, whereas fresh spores may require a number of substances to trigger the germination, after aging, some of the nutrients may be needed in reduced amounts or not at all. We believe that these changes are due to autolytic enzymes which slowly release substances that can function as germination nutrients. In fact, we, as well as others, have shown that during aging, small amounts of alanine are released from the spore, and that this is one of the key substances needed for germination.

In the study of bacterial spores, we are making progress toward an understanding of the mechanisms that are involved in conferring dormancy on cells. We are also learning how to break this dormancy. Further advances in this area may help us to better understand the mechanisms involved in dormancy in other areas of biology.

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# ANHYDROBIOSIS — A MODEL OF A CRYPTOBIOTIC STAGE

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The term anhydrobiosis, a state of ametabolic latent life due to dehydration was introduced by Giard in 1894[1] as an addition to the terms osmobiosis, anoxybiosis, and cryobiosis.

The process of lyophilization or freeze-drying discussed in this lecture is actually a combination of cryobiosis, anoxybiosis and anhydrobiosis, since the micro organisms are first frozen, then oxygen, air and water are removed by vacuum and a dehydrated viable product is obtained.

The problems of anhydrobiosis in higher animals and plants (rotifers, nematodes, fungi, seeds of plants) are the subject of other lectures in this conference; some of them are treated by Keilin in his Leeuwenhoek Lecture[2]. In large animals, the maximal loss of water compatible with viability is about 92%, while in bacteria the loss of 99.5% or more of water is still compatible with continuing existence. Cryptobiosis in spore-forming bacteria is treated in a separate lecture.

In the field of anhydrobiosis of vegetative bacteria, much information is available. In the National Type Culture Collection, out of 2,700 strains which were dried over phosphorus pentoxide, 83% remained viable after 14 years[3]. Freeze-dried staphylococci, pneumococci, streptococci and some Gram negative bacteria were found to be viable after 17[4] and 20 years[5]. Stamp[6] showed that bacteria dried in a desiccator for 2–3 days under reduced pressure preserved their antigens and virulence for at least four years (*S. typhi*, *P. pestis*, *P. leptoseptica*, *E. rhusipathiae*, *S. pyogenes*, etc.).

One of the latest papers on this subject has been published by Feldmann[7], who tested 100 strains of 58 species of bacteria. The bacteria were dried *in vacuo*, or freeze-dried in skim milk, and

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after storage for 6 months and reconstitution were tested for viability, biochemical reactions, serological specificity, virulence, resistance to antibiotics, motility and pigment formation. Of all the strains tested, only three strains of pneumococci, one strain of meningococcus, one strain of *N. gonorhoeae* and four strains of *H. influenzae* were lost. All the viable strains preserved their characteristics very well.

Since the most efficient way of preserving micro-organisms in a desiccated state involves a freezing stage, we will briefly consider what happens to the micro-organisms when they are frozen.

Experiments of Becquerel[8] showed that bacteria withstand temperatures as low as 0.05° K. At very low temperatures (—190°–—272°) all cell constituents are vitrified and dissociation and ionization are suppressed. At —200° chemical reactions are eight million times slower than at 20°. The molecular state of some substances such as peroxides, catalase, hemoglobin and cytochrome changes; Keilin[9], using this phenomenon of change in adsorption spectra in frozen material, found that the spores of *B. subtilis* contained 6% of the cytochrome content of vegetative cells, a fact which he was not able to determine by measuring the respiratory activity of the spores.

Practically, such low temperatures (below —196°) are not employed for the preservation of viability in micro-organisms; rather they are frozen and kept at temperatures around —80°.

It has been shown by Weiser and Osterud[10] that death by freezing involves two processes, (a) a rapid or immediate death caused by freezing and thawing *per se*, and (b) a 'storage death' which is a function of time and temperature. Immediate death occurs at a stage of freezing when extracellular ice formation is being completed. As may be seen in Fig. 1, the rate of storage death is not uniform; it is more rapid at temperatures above —30°, probably because the eutectic points of the solutes in media used for freezing are around this temperature.

Death of cells due to freezing was attributed by Haines[11] to irreversible changes in the bacterial proteins, leading to their

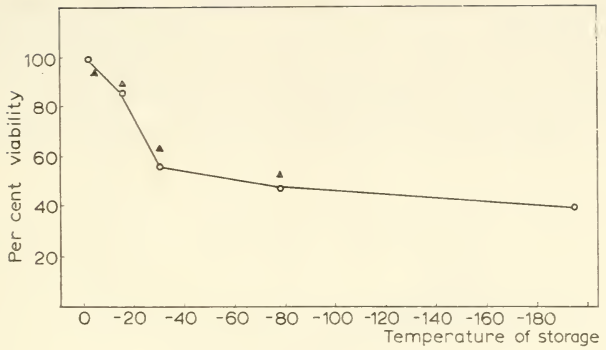


Fig. 1. Storage death of frozen cells of *E. coli*. 15-h culture of *E. coli* in 1 % peptone at pH 7.0, shell frozen in 0.1-ml quantities. –1.5° and –5° samples frozen at –15° then transferred to storage at required temperatures. Other samples frozen and stored at temperatures indicated. Storage time 3–5 h. (Modified from Weiser and Osterud[10]).

flocculation. He found that a protein extracted from *P. pyocyanea* coagulated when stored at —2°, but no such effect was observed when the protein was stored at —20°.

According to Straka and Stokes[12] freezing produces several effects: it kills outright some of the cells, causes damage or reversible injury to others, while some cells entirely escape harm. The injury to bacteria may be detected by the ability of thawed cells to grow in a rich nutrient medium, but not in a minimal medium in which control, non-frozen bacteria grow well. At storage temperatures near 0° a higher percentage of cells actually die than at —30°, when less bacteria are killed and more only injured; the injured ones can be saved on thawing by seeding them into a rich medium. The factor in the medium which was found to be responsible for the repair of the damage was trypticase (Baltimore Biological Laboratory), or rather some peptide in it. Amino acids and vitamins could not replace trypticase.

Our own investigations of damage to the bacterial cell wall

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due to freezing of *E. coli*, as indicated by lysozyme sensitivity, showed that the cells are lysed by lysozyme immediately upon thawing to an extent many times greater than that of cells incubated at 37° for a short period of time after thawing (Fig. 2)[13]. It could further be shown that following this immediate

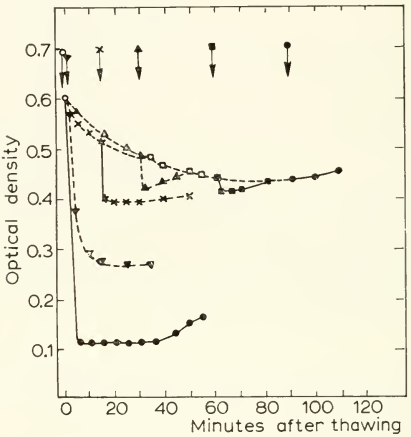


Fig. 2. Loss of sensitivity to lysis by lysozyme of *E. coli* at different times after thawing. At times indicated by arrows 30 $\mu$g/ml of lysozyme was added. Dotted line indicates control lysis up to the time of addition of lysozyme. Each determination made in a separate tube from the same culture. Optical density measured at 540 m$\mu$/(ref.[2]).

recovery after thawing, considered to be osmotic or mechanical, there was a period of metabolic recovery which could be inhibited by starvation or metabolic poisons such as KCN, but not by chloramphenicol or 2,4-dinitrophenol (Fig. 3).

Immediate damage to cells by freezing may be prevented by adding to the bacterial suspension 'protective colloids' such as skim milk. The explanation offered for the action of these colloids is that they prevent the death of bacteria by crushing due to extracellular ice when the inter-crystallic water films are thin. Colloids increase the thickness of these films and thus

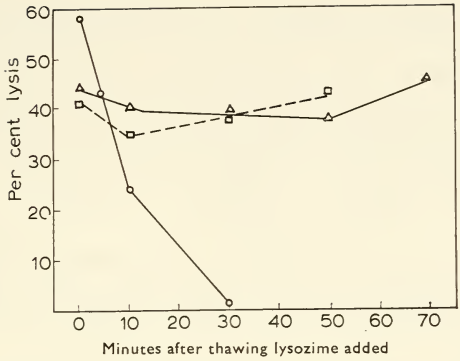


Fig. 3. Sensitivity to lysis by lysozyme of frozen and thawed *E. coli*, following starvation or KCN treatment. *E. coli* starved by aeration in phosphate buffer for 20 min before freezing (△———△), or 0.005 *M* KCN added 20 min before freezing (□———□). Each point represents % lysis in 20 min due to lysozyme added after thawing, at times indicated [2].
Control: O———O.

leave enough space to accomodate the bacteria and prevent them from being crushed. We shall see later that these colloids have no action in protecting bacteria from death due to drying during the process of freeze-drying or after it.

A very important chance discovery made first by Rostand in 1946, and later, independently by Parkes[14], was that the addition of glycerol to animal cells (fowl sperm) protected them from the injurious and lethal effects of freezing. Bull semen has been successfully preserved in the frozen state for several years and calves have been produced by semen from a bull that had died three years previously. Thus a practical method was devised for freezing and storing semen, red blood cells, tissue culture cells and lately bone marrow cells. Recent information[15] indicates that bone marrow cells suspended in glycerol (10%) or frozen in glycerol are much more resistant to ionizing radiation than in the absence of glycerol. The effect of glycerol may be compared to the effect of drying since glycerol is a dehydrating agent.

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Among the many processes of drying or dehydration of micro-organisms, freeze-drying has proved to be the least lethal and injurious. It is used nowadays not only for the storage of bacterial and viral strains but also for the preparation of living vaccines for prolonged storage, for preservation of some tissue used in transplantations, of plasma and of many labile biological materials such as enzymes, antibiotics, hormones, etc.

In the process of freeze-drying of micro-organisms, a suspension of bacteria in a suitable medium is frozen (usually in a shell form on the walls of an ampoule) by dipping the ampoule into a carbon dioxide bath or into liquid air, and the water is then removed from the frozen material by sublimation in a vacuum apparatus. The rate of evaporation of water is such that the energy required for it is provided by the drying of the material, and thus the material remains frozen as long as any water is left in it. When the material is dry, the ampoule may be directly sealed *in vacuo*, or it may be filled before sealing with an 'inert' gas such as hydrogen or nitrogen.

This process of lyophilization,—based on the discovery of Wollaston[16] in 1813 that water may be removed from ice by sublimation,—was first applied practically by Shackell[17] in 1909 and later introduced for large scale preservation of biological materials by Flosdorf and Mudd[18, 19]. The optimal conditions in freeze-drying were extensively studied by Fry and Greaves[20] and Fry[21]. An important contribution to the development of a practical suspension medium was made by Naylor and Smith[22], and their medium, composed of thiourea, ammonium chloride, sodium ascorbate and dextrin, is now widely used.

Various workers have found empirically suspension media in which micro-organisms were well preserved in a dried state and remained viable after prolonged periods of storage. These media range from skim milk to serum and a mixture of serum and glucose and to Naylor's medium.

Various theories have tried to explain why these particular substances protect bacteria undergoing drying, but none of them embraces all the materials involved or explains the results.

Recent investigations of Lion[23] represent a great advance in the understanding of the process of freeze-drying and the importance of various factors involved. His results and their interpretation give a coherent picture of the process and present a reasonable hypothesis which not only accounts for most of the seemingly unrelated findings reported in the literature, but also allows the prediction of new lines of research and practical applications.

The basic questions which Lion asked were: when and why do bacteria die during freeze-drying or during subsequent storage? What agent is lethal to micro-organisms during this process?

To discover the lethal agent he first used 'naked' bacteria, *i.e.* bacteria suspended in distilled water, so as not to obscure the picture of possible protection by any of the chemical compounds present in the usual media. Most of the work was

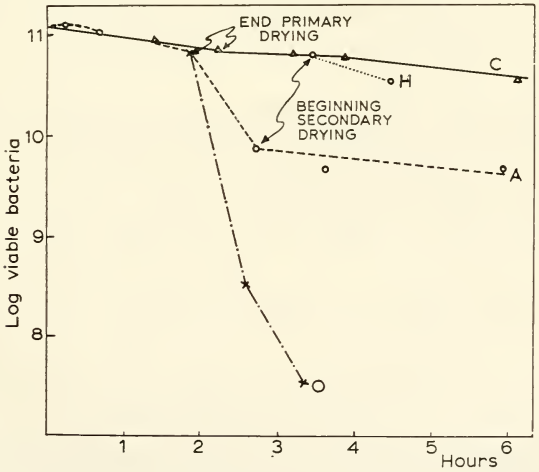


Fig. 4. Kinetics of death of cells of *E. coli* during and following freeze-drying. Washed suspensions of *E. coli* about $10^{11}$/ml in distilled water. Between primary and secondary drying, various gases were introduced into ampoules: C = control in vacuum, H = hydrogen, A = air, O = oxygen.

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carried out on two strains of *E. coli*, which were osmotically stable enough to withstand suspension in distilled water; some experiments were done on other Gram negative bacteria.

He found that the process of drying itself was quite innocuous to bacteria (even though suspended in water only), as long as they did not come into contact with air, or rather oxygen (Fig. 4).

When an inert gas such as hydrogen or nitrogen was introduced into the dried material, the bacteria were preserved in a viable state as if sealed *in vacuo*. The lethal effect of air was proportional to its oxygen content, or to the partial pressure of the oxygen, and there was a reciprocal relation between the pressure of the air acting on the dried bacteria (in the range 50–760 mm Hg) and the time of exposure needed for inactivation.

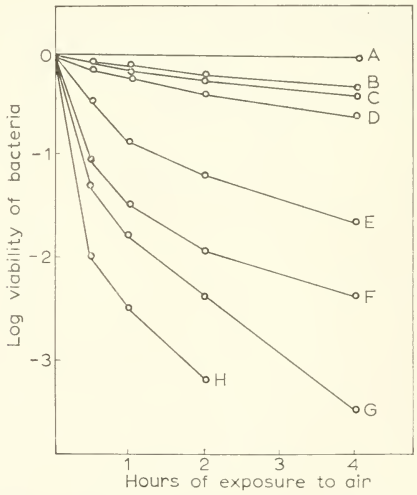


Fig. 5. Protective properties of components of Naylor's medium[22] on freeze-dried *E. coli* exposed to air. 1-ml suspensions of *E. coli* in the following media were exposed to air at the end of freeze-drying. Curve A = control in vacuum; B = full Naylor's medium; C = thiourea 1%; D = thiourea 0.5%; E = ammonium chloride 0.5%; F = sodium ascorbate 0.5%; G = distilled water; H = dextrin 2%.

Lion then investigated the protective properties of Naylor's medium. Using each of its components separately and in combination, he found that only the thiourea protected bacteria from the lethal effects of oxygen (Fig. 5).

Of various analogs of thiourea tested, such as methyl thiourea, dimethyl thiourea, trimethyl thiourea, thioacetamide, urethan, etc., only those in which the amino group was not totally substituted, and which had the S=C rather than the O=C link, had protective powers.

In view of reports on the protective properties of glucose, many sugars, monosaccharides, disaccharides, and trisaccharides were tested. In addition, the protective effects of inorganic salts with different cations and anions were tested, as well as those of reducing agents such as glutathione, cystein, cysteamine, sodium hydrosulfite, etc.

Table 1 summarizes the results. It may be seen that of the inorganic salts, the Na cation, iodides, thiocyanates and nitrites are particularly effective in protecting bacteria against the lethal effect of air. The protective effect of NaI exceeds even that of thiourea.

As to the sugars, their protective properties depended neither on their being fermented by the bacteria (lactose is fermented like glucose, but lacks protective properties), nor on their reducing character (maltose which is reducing but not fermented lacks activity, while $\alpha$-methyl glucoside which is not reducing and not fermented protects very well).

Cystein and glutathione even increased the lethal effect of oxygen while colloids like albumin or dextran had no effect whatsoever.

In another set of experiments it was found that the protective substances may be added as late as ten seconds before freezing, and still prevent death due to oxygen, thus indicating that they have no metabolic effect. They were, however, ineffective when added to the dried bacteria in the resuspending medium after exposure of the organisms to air.

Another very important finding was that the degree of

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TABLE I

PROTECTION OF FREEZE-DRIED CELLS OF *E. coli* AGAINST LETHAL EFFECTS OF AIR (OXYGEN) BY VARIOUS SUBSTANCES

| *Substance* | * *Protective properties* | *Substance* | * *Protective properties* |
|---|---|---|---|
| *Sugars* | | *Salts* | |
| Glucose | 41 | NaCNS | 45 |
| Galactose | 31 | KCNS | 52 |
| Mannose | 42 | | |
| Fructose | 25 | $MgCl_2$ | 36 |
| | | $MgSO_4$ | 0.5 |
| Lactose | 0.04 | $N_2HPO_4$ | 0.01 |
| Saccharose | 0.5 | | |
| Maltose | 0.2 | | |
| | | Water | 0.03 |
| Cellobiose | 1 | Ascorbate (0.5%) | 0.4 |
| Melibiose | 2 | Ammonium chloride (1%) | 2.3 |
| Trehalose | 6 | Dextrin (2%) | 0.002 |
| | | | |
| $\alpha$-Methyl glucoside | 32 | Thiourea (T.U.) (1%) | 42 |
| $\alpha$-Methyl mannoside | 30 | Naylor's medium | 49 |
| | | Monomethyl T.U. | 59 |
| Mannitol | 13 | Dimethyl T.U. (symmetr.) | 39 |
| Inositol | 3 | Dimethyl T.U. (asymmetr.) | 4.5 |
| Glucosamine | 42 | Trimethyl T.U. | 5.5 |
| | | Urea (1%) | 7.8 |
| *Salts* | | Thioacetamide (1%) | 4.7 |
| NaCl 0.16 *M* | 30 | Acetamide (1%) | 0.9 |
| KCl | 0.2 | Thiosemicarbazide (1%) | 0.9 |
| NaBr | 10 | | |
| KBr | 0.4 | $Na_2S_2O_4$ (1%) | $10^{-3}$ |
| | | Cysteamine (1%) | $10^{-2}$ |
| NaI | 80 | Cystein (0.75%) | $10^{-3}$ |
| KI | 82 | Glutathione (0.16 *M*) | $10^{-5}$ |
| | | | |
| $NaNO_3$ | 64 | Nutrient broth (2%) | 0.08 |
| $KNO_3$ ($NH_4Cl$) | 0.7 | Gelatin (1%) | 0.008 |
| LiCl | 12 | Skim milk | 0.03 |
| $NaNO_2$ | 44 | | |
| $KNO_2$ | 68 | Versene (0.1%) | 0.06 |

* Per cent viability of freeze-dried cells of *E. coli* after 4 h contact with air.

protection depended sharply on the proper ratio between the number of molecules of the protective substance and the number of bacteria. When the number of dried bacteria was increased tenfold, optimal viability was achieved when the quantity of the protecting substance was increased in the same ratio. In the case of NaI, approximately 100 million molecules of this compound per bacterial cell gives optimal protection against the lethal effects of air.

An attempt was made to fit the heterogeneous data into a coherent hypothesis. It is clear that oxygen does not destroy the dried bacteria by oxidation or the formation of peroxides, since the protecting substances are mostly non-reductants. Moreover, some reducing substances with SH-groups, like glutathione, even enhance the lethal effect.

Because of the electronic structure of the $O_2$ molecule, we can assume that it is readily reactive with a number of metastable reactive metabolites. The latter may indeed be vital to the cell but ordinarily they are protected from direct attack by dissolved oxygen by the circumstance that oxygen has a low solubility and diffuses in water much more slowly than in a solid phase. Moreover, active metabolism of the cell makes the direct contact of the intermediates with oxygen unlikely. Little more can be said at this stage about the nature of these intermediates. However, it should be mentioned that Commoner detected free radicals in lyophilized biological materials[24]. Also artificial free radicals in proteins produced by high doses of X-rays have been found to be stable *in vacuo* but to react quickly with oxygen and usually to disappear[25]. It may be possible that these radicals are molecules in a triplet state as suggested by Szent-Györgyi[26]. He claims that free energy in metabolic processes passes from place to place through substances in the reactive, triplet state (*i.e.* having electrons with 2 parallel spins). This state is metastable and its life span depends on the conditions prevailing. For instance, the presence of crystalline water or of hydration water around proteins makes it easier for the molecules to pass from singlet to triplet, *i.e.* to a more reactive state in which they

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would react more easily with oxygen. In Szent-Györgyi's experiments, riboflavin phosphate which fluoresced in the presence of oxygen ceased to do so when certain substances, known as fluorescence quenchers, were added. These protective substances —KI, thiourea, cyanates, nitrites, etc.—which deactivate the reactive state sensitive to the magnetic field of oxygen are indeed the same that protect dried bacteria against the lethal effect of oxygen.

Why are cations such as $Na^+$ and $Li^+$ protective, while $K^+$ is not? Since the presence of crystalline water makes transition to the triplet state more probable, any ion which interferes with the formation of the crystal lattice should be protective, as indeed is the case with $Na^+$. In the case of cystein and glutathione, which increase the lifetime of the metastable and reactive states, one would expect enhancement of the oxygen effect, as is actually observed.

High mortality sometimes obtained on drying bacterial suspensions of low density in isotonic solutions may be obviated by adding more cells, alive or dead. Moreover, the same effect is produced by adding certain proteins, *i.e.* 'protective colloids'

TABLE II

PROTECTIVE PROPERTIES OF SODIUM IODIDE AND ALBUMIN ON LYOPHILIZED DRY CELLS OF *E. coli* EXPOSED TO AIR[23]

| *Conc. NaI* % | *Concn. of bacteria before drying* | *Relative viability %* | | | |
|---|---|---|---|---|---|
| | | *No additives* | | *With 4% serum albumin* | |
| | | *A** | *B** | *A** | *B** |
| 1.75 | $8.9 \cdot 10^9$ | 0.02 | –0.002 | — | — |
| 0.175 | $1.5 \cdot 10^{10}$ | 60 | 50 | 47 | 0.3 |
| 1.75 | $1.7 \cdot 10^{11}$ | 71 | 69 | 30 | 11 |
| 0.175 | $1.9 \cdot 10^{11}$ | 60 | 0.7 | — | — |

A* At the end of drying.
*B** After an exposure to air for 220 min at 28°.

such as albumin (Table II). This finding also explains the conflicting results obtained with various protective colloids and different bacteria when unequal concentrations of bacteria were used for drying.

As has been mentioned previously, the 'protective colloids' perhaps diminish the number of deaths due to freezing alone: but if the optimal proportion between bacterial cells and their protective solution, for example NaI, has been established, the addition of the colloid may only make the situation worse.

In conclusion I should like to mention some possible practical applications of these findings concerning the lethal effect of oxygen on dried bacteria.

Analysis of the curve of inactivation of dried cells of *E. coli* by molecular oxygen or air (Fig. 6) indicated that the killing effect was not a monomolecular reaction and that it resembled

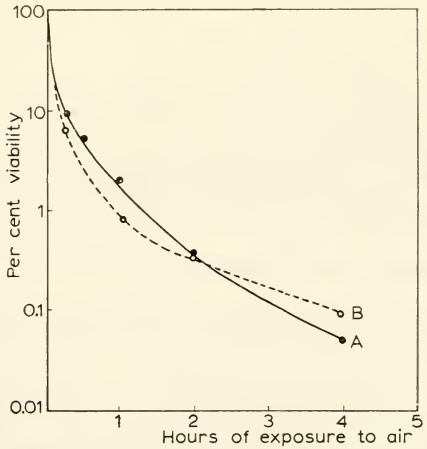


Fig. 6. Kinetics of inactivation of freeze-dried *E. coli* exposed to air. Ampoules containing 1 ml of aqueous suspensions of *E. coli*, B/r ($1.5 \cdot 10^{11}$/ml) were exposed to air at the end of freeze-drying, for various periods of time. Curve A = experimental results; curve B = calculated on the assumption that as regards its lethal effect the concentration of oxygen is reciprocal to the time of its action on dried bacteria (see text).

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very much the curve of inactivation of the virus of poliomyelitis by formalin[27]. In both cases the lethal agent (oxygen or formalin) is in excess and its concentration does not change during the process of inactivation; in both cases the relative mortality of the viruses or bacteria is independent of their initial concentration.

Gard's empirical formula that may be fitted to the curve of inactivation is

$$\ln \frac{y_0}{y} = a \ln (1 + bt) \qquad (1)$$

where $y_0$ is the viability of the virus at time 0, $y$ the viability at time $t$ and $a$ and $b$ are constants. For infinite time of reaction, a plot of log *viability of cells* against log *time* gives a straight line. If $F$ is the concentration of formalin and $C$ a constant, the kinetic equation derivable from the above would be of the form

$$\frac{-\mathrm{d}y}{\mathrm{d}t} = K.F.y$$

where $K = \dfrac{K_0}{1 + CFt}$ and is a function of time. In the case of poliomyelitis, the change of the inactivation constant is explained by a change in resistance of the virus to the action of formaldehyde in the course of the treatment, presumably because of change in the permeability of the virus membrane. As regards oxygen, one may postulate the existence in the bacterial cell of several sensitive targets (the metastable metabolic intermediates) that have different inactivation constants with regard to oxygen, the reaction of oxygen with a single molecule being of first order. The velocity of reaction between the cell and the oxygen would be proportional to the number of targets that have not yet reacted. The reaction constant, although fundamentally of first order, becomes dependent on time, as more and more targets (part of them vital) are knocked out.

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## DISCUSSION

Avi-Dor: Some observations made in our laboratory by Mrs. Miller might be of interest here. When *E. coli* cells were suspended in distilled water, they swelled and lost potassium without losing sodium ions at the same rate. On addition of an oxidizable substance like glutamate no respiration occurred in these cells until potassium was also added. Addition of sodium

did not have the same effect. Potassium iodide was more effective than several other potassium salts. The potassium effect depended on the concentration of bacteria.

KOHN: Dr. Avi-Dor mentioned the metabolic importance of potassium and sodium, and that this effect required a certain time to manifest itself. In our laboratory Dr. Lion has performed a number of experiments in which addition of salt was made one second before freezing. Nevertheless the protective effect was obtained. Regarding the ion effect reported by you, is it a minimum effect or do you get an optimum curve? With glucose the viability rises with concentration until a certain maximum viability is reached. Further additions of glucose do not alter the viability. With thiourea and sodium iodide, however, once you have reached the optimal concentration, further addition decreases the viability rapidly.

AVI-DOR: Regarding your first question: we measure respiration which is a secondary effect of potassium. We do not know whether the primary effect is instantaneous or not. As to your second question, we did not reach an inhibitory concentration with potassium.

MAYER: Have you tried to correlate the fluorescence quenching action of thiourea derivatives and their protective action? What is your evidence that free radicals are involved? Does an increase in thiourea concentration protect against a higher oxygen concentration? Finally has dinitrophenol any effect?

KOHN: Only three thiourea derivatives were found to be active: thiourea, monomethyl thiourea and symmetric dimethyl thiourea. Regarding the relation between oxygen concentration and that of thiourea, no specific experiments were carried out.

SHILO: In some bacteria, some unicellular algae and certain higher plants carotenoid pigments protect against photo-oxidative death. It occurs to me that a comparison between bacteria containing carotenoid pigments and mutants lacking

carotenoid would be useful as a means of clarifying the mechanism of the oxygen effect.

HESTRIN: Is the oxygen-death a temperature-dependent reaction? By the way, if the specific target is inside the cell and the salt in question is outside, how does the interaction come about in the solid state?

KOHN: In regard to your first question: when the dried ampoule is opened at —80° and kept at that temperature the lethal effect does not occur. As to your second question, there could be a steric effect involving crystalline water and protein. The substrate acted upon may be a part of the electron transport system of the cell.

# THE BACTERIAL ENDOSPORE

## A BRIEF REVIEW OF BIOCHEMICAL ADVANCES AND SOME NOTES ON SPOROGENESIS

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Three crucial observations have, in my opinion, stimulated many of the recent researches on spores. One, the observation by Hills[1] that spores will germinate rapidly in the presence of a mixture of amino acids and nucleosides; two, the discovery in our laboratory that spores contain active heat-resistant enzymes[2–4] and three, the observations made by Powell[5], that dipicolinic acid is a major constituent of spores. I want to take some time to discuss each of these observations.

From early studies it was known that spores suspended in a growth medium would lose their heat resistance in a relatively short time. In looking into this further, Hills[1] found that yeast extract had the same effect. He then proceeded to fractionate this to determine what nutrients were responsible for this effect, and discovered that spores would germinate in a few minutes in the presence of a few amino acids, with or without nucleosides and glucose. L-Alanine and adenosine were found to be sufficient for some species. This was studied in more detail by the late Joan Powell[6] *et al.* They found that a large percentage of the spores in a suspension (in the presence of the proper nutrilites) would simultaneously lose their heat resistance and refractility, and at the same time would become stainable. Many investigators have confirmed these findings since they were made early in 1950. In addition, the minimal germination requirements for other species have been determined. In most cases germination can be initiated by a few amino acids along with nucleosides, although some species will germinate with glucose only.

As a result of the studies on the germination requirements, we have also gained a fairly good insight into the effect of environ-

mental factors upon germination. It is necessary, at this point, to indicate that germination is used here in a somewhat different sense than it has commonly been employed in the past. In older literature the word has been used to describe all of the changes taking place during the development from spore to fully grown vegetative cell. The changes observed by Hills and later by Powell and other workers occur very early in the process, and bacteriologists have come to use the term to encompass these early changes only, and to use the word *outgrowth* to represent all the other changes.

A number of investigators have shown that many species of spores require a heat shock as a sensitizing mechanism before they will respond to germination nutrients. The length of the time of heating and the temperature required vary with different species, the age of the spores, and the conditions of storage. Freshly grown spores of *Bacillus cereus* can be adequately sensitized by heating to 65° for about 15 min. On the other hand, spores of *Bacillus stearothermophilus* have to be heated to 100° or more for an equivalent length of time[7]. Most workers in the field believe that this heat sensitization initiates biochemical reactions that either release compounds to stimulate germination or alter the permeability of the spore wall. In any event, precise information as to what actually does happen is not available.

The rate of germination is notably influenced by the temperature. Most of the investigators have been observing germination at room temperature, and it has been their common observation that it will not take place in a refrigerator or at a lower temperature, nor will it occur at 60°[3]. On the other hand, Wynne[8] claimed that spores from some species of anaerobes germinated at 75° when they were suspended in a solution containing glucose that had been autoclaved in an alkaline medium. Recently, Foster[9] reported that *Bacillus megatherium* spores will germinate at temperatures between 70° and 100°. In our own laboratories, we find spores of *B. cereus* will germinate rapidly in the presence of L-alanine and adenosine at room

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temperature but will not do so in the same menstruum if kept at 65°.

The germination requirements are also affected by aging. Freshly produced and thoroughly cleaned spores have the most rigid requirements, but as they are aged the requirements generally become less so. The rate at which these changes take place depends upon the temperature of storage. Spores stored in a frozen state can remain unchanged for a very long period, whereas those stored in a refrigerator will change more rapidly. There is a marked difference in different species as to the rate at which these changes can take place. One of the most noticeable effects of aging is the disappearance of the need for heat sensitization. The changes that take place during aging are probably the same type as those which occur during heat shock. The only difference is one of time. In our laboratories, we have detected free L-alanine in the same supernatant liquor in which spores have been stored and aged, but this amino acid cannot be found in the supernatant liquor from freshly prepared clean spores[10]. Alterations probably occur within the spore during aging, resulting in the release of chemicals required for germination. It is for this reason that aged spores will germinate with a lower concentration of L-alanine than fresh spores, and in some cases they will germinate with alanine alone and no adenosine, or in other cases with adenosine alone and no L-alanine.

Various kinds of metals have a marked effect upon germination. Some metal ions, such as cobalt and nickel, will inhibit the process, whereas calcium and magnesium ions are helpful[11]. We encountered this phenomenon in a batch of spores which had been produced for us in a pilot plant where the fermentors were made from metal. The spores we obtained from this pilot plant failed to germinate unless we added to the suspension some chelating agent such as versene or heavy concentrations of phosphate[12]. Observations made by Brown[13] are of interest in this connection. He found they could germinate spores of the putrefactive anaerobe 3679 with versene only. In this case, it appeared that these spores could germinate spontaneously,

except for the presence of some metal ions which inhibited the process. Upon the addition of versene, the toxic metal ions were removed and germination proceeded. Dr. Riemann[24] (personal communication) in Denmark, working in Ordal's laboratory, has made an even more interesting observation. He has been able to germinate most spores with an equimolecular mixture of calcium and dipicolinic acid. He observed this while making some studies on the effect of chelating agents on germination. Knowing that dipicolinic acid is a fairly effective chelating agent, he tried to use this in place of versene and found it would bring about germination only in the presence of calcium ions. The most rapid change was obtained when he had an equimolecular mixture of the two.

It is apparent from the above that the discoveries made by Hills can be looked upon as forerunners of many important advances in our knowledge of the germination of spores.

The second observation which I mentioned above, namely, the demonstration of active enzymes in intact spores, was made in connection with some of our studies on germination. We observed, as others have, that when spores germinate in the presence of L-alanine and adenosine or, as a matter of fact, with any of the other combinations of germination ingredients, not all of the spores in a suspension will do so. From 90 to 98% germinate, but the balance remains heat-stable and unchanged. The question naturally arises, why do these remaining spores fail to behave like the rest? Is it because they are different or because something has happened to the menstruum? To answer this question we examined the solution in which the spores had germinated to see whether the L-alanine or the adenosine, or both, had been used up. We found there appeared to be no change in the concentration of either, during the germination process. If either of the chemicals had been used, the amount was too small to be detected by our methods of analysis. It appeared nothing had happened to the solution; one would be tempted to assume, therefore, that the spores which failed to germinate might be different from the remaining bulk that

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germinated. In order to throw light upon this problem, we centrifuged the suspension at the conclusion of the germination and added to the supernatant some fresh spores, but none of these germinated. This clearly demonstrated that something had happened to the menstruum. Since there was no change in the total amount of alanine present, we examined the L-alanine to see if some had been converted to D-alanine. This appeared reasonable since Hills[14] had previously demonstrated a D-alanine interference with germination brought about by L-alanine. A racemic mixture of L and D was found after less than one hour's contact with the spores. This prompted us to look for the enzyme racemase which, fortunately, was very easily found[4]. It proved to be an interesting enzyme because it was active in the intact spore and was heat resistant, withstanding temperatures up to 100°. Upon more careful study, we found the enzyme was not only heat resistant but remained so even after the spores germinated. Furthermore, this enzyme was attached to some colloidal particles. Upon separation from the carrier by means of a sonic oscillation, it becomes heat sensitive.

These observations stimulated us and others to look for more enzymes in spores. Previous to this time, most bacteriologists had assumed that spores were devoid of enzymes because most studies made in the past had resulted in negative findings. A variety of enzymes similar to the racemase have now been found in the spores of aerobic bacilli[15, 16]. A heat-resistant catalase is present in most aerobic spores and also a heat-resistant ribosidase, an enzyme which hydrolyzes adenosine into adenine and ribose. The latter enzyme has also been shown to be heat resistant and to be associated with a colloidal particle. The heat-resistant catalase does not appear to be particulate. There is strong evidence to suspect its existence in spores of other heat-resistant enzymes, particularly proteolytic enzymes[17]. These may, in fact, be the ones responsible for the changes occurring during storage and also during heat shock; for if these are like racemase, they will not be destroyed by the temperatures used for heat sensitization, and the reactions they bring about

may be materially speeded up at these higher temperatures.

A variety of dormant enzymes have since been found to exist in spores, in addition to those mentioned above. These become activated when the spores germinate or are ruptured mechanically. In the dormant state they must be resistant to heat, because such enzymes can be found in spores that are germinated after heating. In fact, it appears from the work of Church and Halvorson[18] that a higher activity may be obtained from extract of spores following heat shock than from unheated spores. Although these enzymes are resistant to heat in the dormant state, they are heat sensitive following germination and mechanical rupture of the spores. The mechanisms which are involved in conferring heat resistance on spores also appear to render them inactive. I shall not attempt to discuss this further since it may be covered in the subsequent lecture.

The third observation which I mentioned above, namely, that spores contain the chemical dipicolinic acid, has also proven to be a very powerful stimulus to researchers of spore physiology. This observation, as well as the one concerning the heat-resistant enzymes, was a natural consequence from Hills' early discovery. Powell *et al.*[6], while studying germination, detected a number of organic compounds which had been secreted during the process. They then proceeded to examine the supernatants from germinated spore suspensions and, thus, discovered that dipicolinic acid along with other materials was released from the spores during germination. Among the other materials were calcium ions and a spore peptide. The occurrence of dipicolinic acid was of special interest because this was the first time this chemical had been reported in a natural product. A follow up of these studies has shown that dipicolinic acid is a normal constituent of all spores of bacteria (both aerobes and anaerobes) and that it is present in considerable quantities, varying from 6% to 12% of the dry weight of normal spores. As soon as these announcements were made, everyone who was interested in spore research examined their spores for this chemical and were able to confirm the observation of Powell.

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Further studies on this chemical have brought out a number of interesting points in connection with the physiology of the bacterial spore. Nearly all the dipicolinic acid is released into the outside medium when the spores germinate. The release of this acid correlates almost perfectly with the loss in heat resistance, the loss in refractility, and the gain in stainability; and this provides good circumstantial evidence that dipicolinic acid plays an essential role in the unique heat-resistant properties of spores. Studies which have been made on the activation of the dormant enzymes through heat shock, germination, or mechanical rupture also show a very good correlation between the release of the dipicolinic acid and the activation of these enzymes[12]. This gives further circumstantial evidence for the importance of dipicolinic acid in the protection of these enzymes in the intact spore. The acid is also released from the spores when they are killed by heat[19]. This fact has been demonstrated in a number of laboratories. The temperature that is required is dependent upon the heat tolerance of the spores themselves. Thus, the spores of thermophilic organisms must be heated to a higher temperature to release the dipicolinic acid than those of some less resistant aerobic organisms. In a recent announcement by Foster[9], he reports that dipicolinic acid can be released from spores of *B. megatherium* at temperatures ranging from 70° to 100°. As the temperature is increased, less time is required.

These numerous observations have led investigators in this area of study to believe that spores are made heat resistant and their enzymes protected by a complex collodial structure involving a polymer formed from dipicolinic acid, calcium, and special peptides. It would indeed be interesting to know more about the nature of this complex. So far, it has remained obscure because no means has yet been found to rupture the spore and retain the complex. Any form of mechanical rupture breaks up the complex and releases the dipicolinic acid in the same way as germination does. The breaking of this complex during germination may well be an enzymatic process and the enzymes may be activated by mechanical rupture as well, so that

when spores are broken up by mechanical means, the complex is again decomposed through the same mechanism that occurs during germination.

The ultimate objective for most of the investigators on bacterial spores is to explain the means by which these structures gain heat resistance. To us it appears we have reached an impasse in our attempts to unravel this mystery by a study of the germination process. We have, therefore, for the time being at least, suspended our studies on germination and focussed our attention on the process of sporulation, hoping this may be a more fruitful study for us. I will devote the rest of this discussion, therefore, to a report on some recent observations we have made on sporulation.

Although the bulk of this report is going to be based upon studies made on *Bacillus cereus*, an aerobic sporeformer, the investigation had its beginnings in studies made in our laboratory on the anaerobe *Clostridium roseum*[20]. When we first began the study on the anaerobe, we were unable to obtain a good yield of spores. This was due to a recycling phenomenon occurring in the culture. Spores that were produced early in the growth cycle would germinate in the same stew in which they were produced. This resulted in a culture having cells in all stages of development including freshly germinated spores, actively growing vegetative cells, sporulating cells, and spores just released from their sporangia. In such a mixture it was virtually impossible to isolate clean spores. To overcome this difficulty we developed a technique of growing the organisms under semi-synchronous conditions. This was done by inoculating the medium in which the spores were to be produced with a very heavy inoculum from an actively growing synchronous culture. The result was a population in which nearly all of the cells began to produce spores at about the same time. We were thus able to harvest clean spores containing a minimum of vegetative cells and freshly germinated spores. In such cultures we found sporulation set in very early; in fact, the process was complete in about seven hours. By staining an hour or so before

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any dipicolinic acid was synthesized, we obtained what appeared to be normal spores. Furthermore, the synthesis of dipicolinic acid was complete, or nearly so, before any of the spores had developed heat resistance. Heat resistance developed approximately an hour after the synthesis of DPA. The development of a spore-like structure (this study would indicate) occurs independent of the synthesis of DPA and this precedes the development of heat resistance. This is shown graphically in Fig. 1.

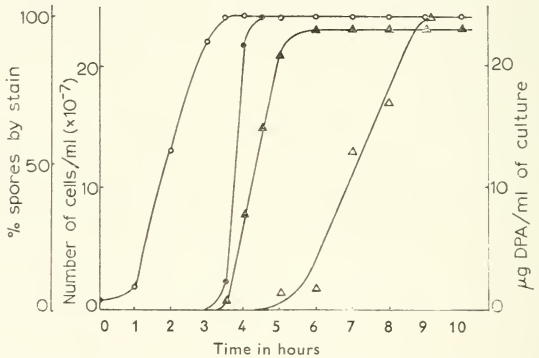


Fig. 1. The relationship of heat resistance–total viable count–synthesis of DPA in *Clostridium roseum* at 37°. ○ = Total viable count; ● = % spore-stain; △ = DPA synthesis; ▲ = heat resistant count.

When we attempted to repeat this with the aerobe *Bacillus cereus*, our cultures would lyse about the time they should be producing spores. Investigation showed this was due to a lack of oxygen[21]. By using a very heavy inoculum, we developed a condition in which the demand for oxygen exceeded our ability to dissolve oxygen in the water. This prompted us to make a study of the oxygen demand of cultures during various stages of growth and sporulation. The results of this study are shown in Fig. 2. This shows that the oxygen demand curve is bimodal. The first peak in this curve occurs about the time the maximum population of vegetative cells is reached. I should mention that

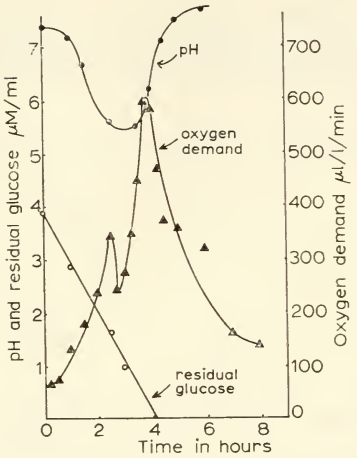




Fig. 2. pH, oxygen demand and residual glucose *vs.* time.

the oxygen demand was determined by measuring the rate of disappearance of dissolved oxygen from an aliquot sample of the culture by means of a dropping mercury electrode and a polarograph. By making frequent measurements of the dissolved oxygen content in such a sample, we could plot a curve showing the change in concentration of dissolved oxygen *versus* time: and the slope of this curve, we considered the measure of the oxygen demand.

As is apparent from the above, we reached the first peak in the oxygen demand curve at the same time we obtained the minimum in the pH curve. Shortly thereafter, the pH begins to rise and with this rise a very sharp rise in the oxygen demand occurs. A new enzyme system has apparently developed to utilize the acids which are responsible for the lowering of the pH. As these acids are oxidized, a very high demand for dissolved oxygen develops. In fact, the oxygen demand at this stage far exceeds the oxygen demand during vegetative growth. During this second rise in the oxygen demand curve and the corresponding rise in the pH curve, we began to observe

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morphological changes in the rods which were typical of presporulation. Spores themselves do not actually appear until much later. The actual time of onset of sporulation is shown in Fig. 3.

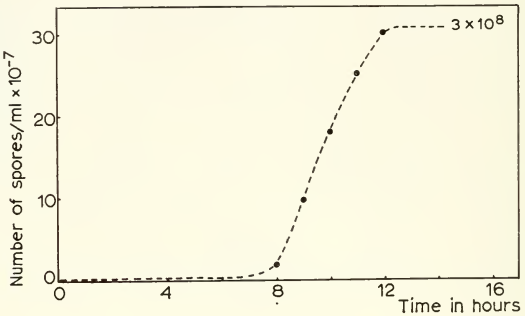


Fig. 3. Time of sporulation in active culture of *B. cereus* T.

We also investigated the nature of the acids which are released during the early vegetative cell growth and found that only two acids formed, pyruvic and acetic[21]. The pyruvic acid appears first and is subsequently converted to acetic acid. This is illustrated in Fig. 4 and 5. These acids appear quite stable during the period of vegetative cell growth, but begin to disappear as the pH begins to rise and, presumably it is the oxidation of the acetic acid that creates the high demand for dissolved oxygen just preceding sporulation. Our failure to obtain spores in our initial experiment was because the air supply was not sufficient to satisfy the oxygen demand in this stage of the development of the culture. This difficulty can be overcome in one of two ways; by improving the efficiency of aeration, and by reducing the concentration of the glucose so that less acid is formed and consequently less oxygen is needed. This latter course also reduces the final spore crop. The metabolic processes going on during vegetative cell growth must, therefore, be quite different from those which take place in the

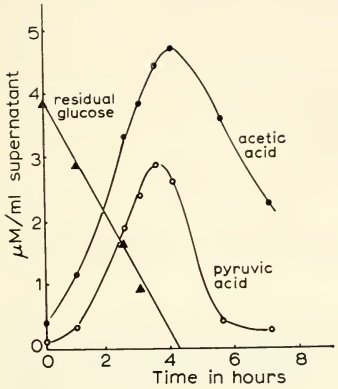


Fig. 4. Pyruvic and acetic acid production *vs.* time.

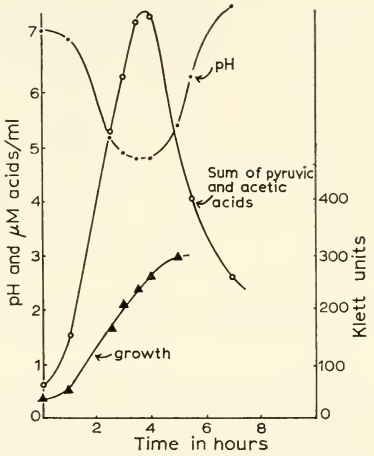


Fig. 5. pH and sum of pyruvic and acetic acid *vs.* time.

culture just preceding sporulation. During the growth of the vegetative cells, the glucose is broken down to acids, but the acids are not utilized. When the glucose is completely gone, an adaptive enzyme apparently forms for the utilization of the acids. This accounts for the rise of the pH curve. One may also assume that the oxidation of these acids is supplying the energy needed for the synthesis of the spore material.

These observations led us to investigate a variety of inhibitors to see if compounds which would inhibit the utilization of intermediates might also interfere with sporulation. The first one we studied was $\alpha$-picolinic acid[22]. It is obvious why we selected this one. We believed it might serve as an analogue for dipicolinic acid and might interfere with its synthesis and thus interfere with the production of the spores. The results obtained with this inhibitor were quite surprising and are shown in Fig. 6. An examination of this figure will show that $\alpha$-picolinic acid, when added to the culture while the pH is still dropping, interferes

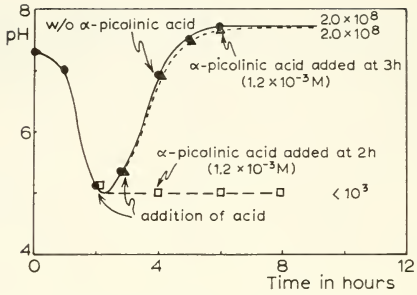


Fig. 6. Effect of dipicolinic acid on the sporulation of *B. cereus* T.

with the subsequent utilization of the acids, while the pH remains low and no spores result. If this material is added to the culture after the pH begins to rise, there is no effect; the pH rises normally, and the result is a normal spore crop. This inhibitor may, therefore, interfere with the development of the adaptive enzyme required for the utilization of the acetic acid.

In view of the unexpected result with $\alpha$-picolinic acid, we felt we should try other pyridine carboxylic acids. The results are shown in Table I. It is seen from this that $\alpha$-picolinic acid is the only acid able to inhibit sporulation.

TABLE I

EFFECT OF PYRIDINE–CARBOXYLIC ACIDS ON THE SPORULATION OF *B. cereus* T.

| *Compound added* | *Sporulation* |
|---|---|
| None | + |
| Nicotinic acid (Pyridine-3-carboxylic acid) | + |
| Isonicotinic acid | + |
| Quinolinic acid | + |
| Pyridine-2,4-dicarboxylic acid | + |
| Pyridine-2,5-dicarboxylic acid | + |
| Dipicolinic acid (Pyridine-2,6-dicarboxylic acid) | + |
| $\alpha$-Picolinic acid (Pyridine-2-carboxylic acid) | — |

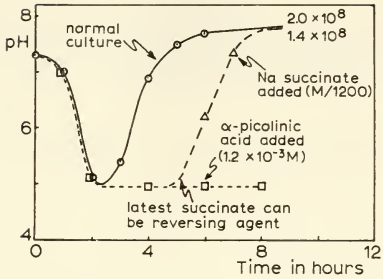


Fig. 7. Reversal by succinate of the inhibition of sporulation of *B. cereus* T. by α-picolinic acid.

In Fig. 7 we show the effect of succinic acid on the inhibition of sporulation by α-picolinic acid. This shows that succinic acid reverses the inhibition, even when it is added as late as five hours after growth starts. In view of this, we have tested a large number of other acids, including some amino acids, for their ability to reverse this inhibition. The results are shown in Table II and Table III. It is to be noted that all of the inter-

TABLE II

EFFECT OF AMINO ACIDS ON THE INHIBITION OF SPORULATION BY α-PICOLINIC ACID

| *Addition of α-Picolinic acid* ($1.2 \times 10^{-3}$ *M*) 1 mg/ml *to 1 mg/ml of* | *Sporulation* |
|---|---|
| Aspartic acid | + |
| Asparagine | + |
| Alanine | — |
| Arginine | — |
| Citrulline | — |
| Cysteine | — |
| Cystine | — |
| Diaminopimelic acid | — |
| Glutamic acid | — |
| Glutamine | — |
| Glycine | — |
| Histidine | — |
| β-Hydroxyglutamic | — |

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TABLE III

EFFECT OF SOME ORGANIC ACIDS ON THE INHIBITION OF SPORULATION BY α-PICOLINIC ACID ($1.2 \times 10^{-3}$ *M*)

| *Compound added to α-picolinic acid* | *Concentration mg/ml* | *Sporulation* |
|---|---|---|
| Formic | 1 | + |
| Malonic | 1 | + |
| Propionic | 1 | + |
| Methyl malonic | 1 | + |
| Pimelic | 2 | + |
| α-Ketopimelic | 2 | + |
| Oxalic | 2 | — |
| Adipic | 2 | — |
| Glutamic | 2 | — |
| Mesotartaric | 2 | — |
| Lactic | 2 | — |
| Pipecolic | 2 | — |
| α-Aminopimelic | 2 | — |
| α-Hydroxyglutaric | 2 | — |
| DL-α-Methyl glutamic | 4 | — |
| Glycolic | 1 | — |
| β-Hydroxybutyric | 2 | — |
| α-Ketoglutaric | 2 | — |
| Fumaric | 4 | — |
| Glyoxylic | 1 | — |
| Pyruvic | 1 | + |
| Acetic | 1 | + |
| Citric | 1.5 | + |
| *cis*-Aconitic | 1.5 | + |
| Isocitric | 1.5 | + |
| Succinic | 0.5 | + |
| Malic | 1 | + |
| Oxalacetic | 1 | + |

mediates in the tricarboxylic acid cycle and the glyoxalic acid shunt reverse this inhibition except fumaric acid, ketoglutaric, and glyoxalic acid. In addition, the inhibition is reversed with a number of other acids. Most of these can readily enter the cycles mentioned. Succinic acid proved to be the best reversing

agent, in that it would reverse the inhibition in concentrations smaller than any of the other substances tested. Among the amino acids, aspartic acid and asparagine are the only ones that were effective.

From the above results we were led to suspect that the glyoxalic acid shunt was the one needed for intermediates for the synthesis of spore protein and dipicolinic acid. By these findings we were encouraged to investigate other inhibitors to see if we could cast further light upon this problem and get further indications as to whether or not the tricarboxylic acid cycle or the glyoxalic shunt are involved.

Before further pursuing this problem we investigated the effect of metals on the inhibition with $\alpha$-picolinic acid, because this compound is a strong chelating agent and its effect may be due to the removal of some essential metal ion. In order to interpret these experiments one needs to know the composition of the medium in which the spores are grown and in which the $\alpha$-picolinic acid is producing its effect. Therefore, I indicate at this point the composition of the medium. This is shown in Table IV. Table V shows the effect of added metal ions on the

TABLE IV

MEDIUM USED FOR GROWTH AND SPORULATION OF *Bacillus cereus* T.

| *Compound* | % |
|---|---|
| $FeSO_4 \cdot 7H_2O$ | 0.00005 |
| $CuSO_4 \cdot 5H_2O$ | 0.0005 |
| $ZnSO_4 \cdot 7H_2O$ | 0.0005 |
| $MnSO_4 \cdot H_2O$ | 0.005 |
| $MgSO_4$ | 0.02 |
| $(NH_4)_2SO_4$ | 0.2 |
| $CaCl_2 \cdot 2H_2O$ | 0.08 |
| $K_2HPO_4$ | 0.5 |
| Yeast extract | 0.2 |
| Glucose | 0.1 |
| Final pH 7.25–7.45. | |

*References p. 59*

TABLE V

EFFECT OF MINERALS ON THE INHIBITION OF SPORULATION OF *B. cereus* T. BY α-PICOLINIC ACID OR VERSENE

| *Addition of α-Picolinic acid with* | *Sporulation* |
|---|---|
| 4 × concn. of minerals | — |
| $Mn^{2+}$ | — |
| $Mg^{2+}$ | — |
| $Ca^{2+}$ | — |
| $Fe^{2+}$ | — |
| $Cu^{2+}$ | — |
| $Zn^{2+}$ | — |
| $Co^{2+}$ | + |
| $Ni^{2+}$ | + |
| Versene (1.5 mg/ml) | — |
| Versene, 2 × concn. of minerals | — |

Concentration of the minerals found in the medium, see Table IV.

inhibition with α-picolinic acid. The only mineral ions that reverse the inhibition are zinc, cobalt, and nickel. It is to be noted that manganese, magnesium, calcium, iron, and copper do not have this effect, nor can we reverse the inhibition by increasing the normal minerals of the medium fourfold. From these data one might conclude that α-picolinic acid does bring about its inhibition by removing some essential ion. If this is so, then the ion must be bound more firmly than the ions of manganese, magnesium, calcium, iron, and copper, but less firmly than zinc, cobalt, and nickel ions. In the light of these results, we also tried versene. It is to be noticed that versene, added to the extent of 1.5 mg/ml, also interferes with sporulation; but the effect of the versene can be overcome by doubling the concentrations of the minerals which are normally present in the growth medium. A general chelating agent such as versene must therefore have a different effect than the α-picolinic acid. If α-picolinic acid is producing its effect through a chelating action, it must have a rather specific effect upon some special mineral. It would be interesting to pursue this further, but in view of other interesting problems we have not taken the time to do so.

As other possible inhibitors of sporulation, we have tried the esters of acids in the tricarboxylic acid cycle. The results are indicated in Table VI. In this table, failure to get spores shows that

TABLE VI

EFFECT OF SOME ESTERS ON SPORULATION OF *B. cereus* T

| *Compound added* | *Concentration* | *Sporulation* |
|---|---|---|
| Ethyl pyruvate | $1.5 \times 10^{-2}$ *M* | — |
| Ethyl acetate | $7 \times 10^{-2}$ *M* | + |
| Triethyl citrate | $2.4 \times 10^{-2}$ *M* | + |
| Diethyl succinate | $2 \times 10^{-2}$ *M* | — |
| L-Glutamic acid diethyl ester | $1 \times 10^{-2}$ *M* | + |
| Ethyl malonate | $1.3 \times 10^{-2}$ *M* | — |
| Ethyl formate | $7 \times 10^{-2}$ *M* | + |
| Diethyl oxalacetate | $1.2 \times 10^{-2}$ *M* | — |
| Ethyl propionate | $3 \times 10^{-3}$ *M* | + |
| None (control) | — | + |

the ester is serving as an inhibitor whereas a normal spore crop shows that no such inhibition takes place. Ethyl pyruvate, diethyl succinate, ethyl malonate, and diethyl oxalacetate inhibited sporulation, but ethyl acetate, triethyl citrate, ethyl

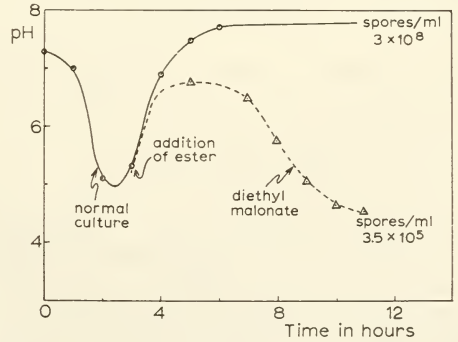


Fig. 8. The effect of diethyl malonate ($1.3 \times 10^{-2}$ *M*) on the pH and sporulation of a culture of *B. cereus* T.

*References p. 59*

succinate, diethyl L-glutamate, ethyl formate, and ethyl propionate did not.

Fig. 8 shows the effect produced by ethyl malonate. It is obvious from this that ethyl malonate behaves differently than $\alpha$-picolinic acid. This inhibitor prevents sporulation whether it is added before the pH begins to rise or afterwards. This inhibitor, therefore, probably does not interfere with the formation but instead interferes with the function of some essential enzyme. With this inhibitor the pH rises for a while as if the culture were normal but finally falls to the low level produced with $\alpha$-picolinic acid. We know from other carefully controlled experiments that the interference with sporulation in this case is not due to a drop in the pH but rather to a specific effect of ethyl malonate.

Fig. 9 shows the effect produced with diethyl succinate as an

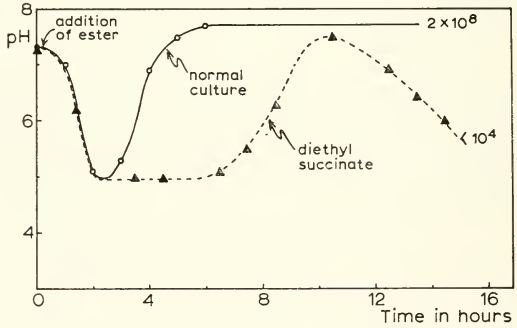


Fig. 9. The effect of diethyl succinate ($2 \times 10^{-2}$ *M*) on the pH and sporulation of *B. cereus* T.

inhibitor. Here again, the inhibition occurs whether the inhibitor is added before or after the pH begins to rise. Here also, as in the case of the ethyl malonate, the pH rises for a while and then drops. Fig. 10 shows the effect produced with ethyl pyruvate. Here also, the inhibitor functions whether it is added before or after the pH begins to rise, indicating that this inhibitor, as well

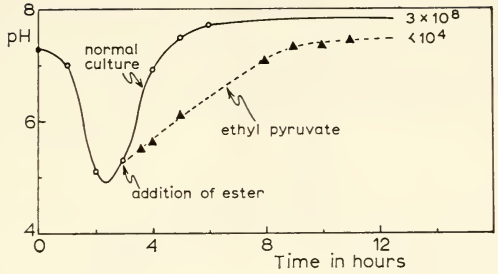


Fig. 10. The effect of ethyl pyruvate ($1.5 \times 10^{-2}$ *M*) on the pH and sporulation of a culture of *B. cereus* T.

as the other two, probably interferes with the functioning of some enzyme systems rather than with the production of an adaptive enzyme. The ethyl pyruvate acts somewhat differently from the two inhibitors cited above, because in this case the pH rises and stays high. Nevertheless, no spores are formed. We have also investigated the effect of various organic acids upon the reversal of inhibition of these ethyl esters. I am not going to take time to discuss the details of all these experiments, but suffice to say, these inhibitors were reversed by all of the intermediates in the glyoxylic acid shunt, but were not reversed by fumarate or other intermediates in the TCA cycle not common to the glyoxylic acid shunt.

We realize it is dangerous to rely upon inhibitors alone for the verification of a definite pathway in a fermentation, but the circumstantial evidence we had for the involvement of the glyoxylic acid shunt led us to conduct further experiments to see if we could get additional support for this conclusion. We therefore investigated two other possible inhibitors. If either the TCA or the glyoxylic shunt (the cycles are shown in Fig. 11) is involved, fluoroacetic acid should also be an effective inhibitor, inasmuch as this material interferes with the enzyme that converts citrate to isocitrate. We found this acid to be an effective inhibitor of sporulation. It did not interfere with the

*References p. 59*

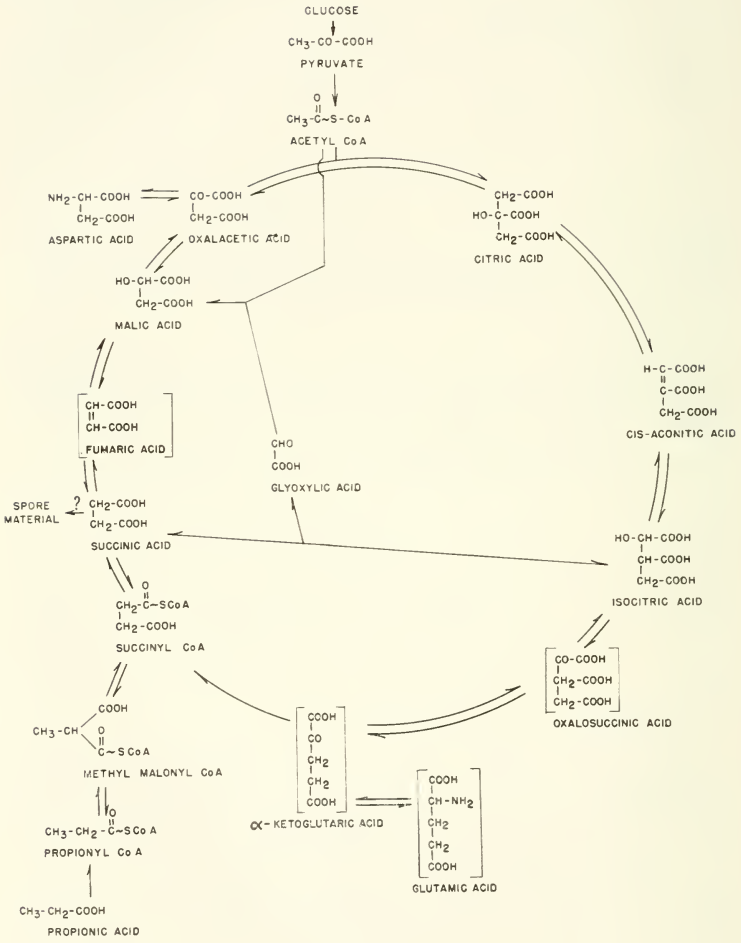


Fig. 11. TCA and glyoxylic and cycle.

growth of the vegetative cells. In this way, it functions very much like the esters we reported above. The action of this inhibitor was reversed by citrate, isocitrate, succinate, and malonate, but not by fumarate, acetate, pyruvate, or $\alpha$-ketoglutarate. This is shown in Table VII.

TABLE VII

EFFECT OF SOME ORGANIC ACIDS ON THE INHIBITION OF SPORULATION BY FLUOROACETIC ACID (FAA)*

| *Addition (final concentration $10^{-2}$ M)* | *Viable cells/ml* | *Heat-stable cells/ml* |
|---|---|---|
| None (control) | $6 \times 10^7$ | $2 \times 10^8$ |
| FAA only | $1.5 \times 10^4$ | < 100 |
| FAA + Acetate | $1.3 \times 10^7$ | $2.6 \times 10^3$ |
| FAA + Pyruvate | $6.6 \times 10^6$ | $1.5 \times 10^4$ |
| FAA + Citrate | $1.4 \times 10^8$ | $1.3 \times 10^8$ |
| FAA + Isocitrate | $2.2 \times 10^8$ | $1.4 \times 10^8$ |
| FAA + $\alpha$-Ketoglutarate | $4.8 \times 10^7$ (Extensive lysis) | $3.8 \times 10^4$ |
| FAA + Succinate | $7 \times 10^7$ | $1.3 \times 10^8$ |
| FAA + Malonate | $6 \times 10^8$ | $1.4 \times 10^8$ |
| FAA + Fumarate | $4 \times 10^7$ | $6 \times 10^4$ |

* An active culture was used and all compounds were added immediately after inoculation.

We also tried sodium bisulfite as an inhibitor, reasoning that, if the glyoxylic acid shunt is involved, bisulfite should tie up the glyoxylic acid because of its aldehyde group, and thus break the cycle; of course, it may also tie up the ketone group of the oxalacetate which is common to both the glyoxylic acid shunt and the TCA cycle. In any event, we found that bisulfite did effectively interfere with sporulation but did not interfere with the growth of the vegetative cells, as shown in Table VIII. This inhibitor was reversed by citrate, *cis*-aconitate, isocitrate, succinate, methyl malonate, malonate, and glyoxylate. It was not reversed by pyruvate, acetate, $\alpha$-ketoglutarate, aspartate or

*References p. 59*

TABLE VIII

EFFECT OF SOME ACIDS ON THE INHIBITION OF SPORULATION BY SODIUM BISULFITE ($4 \times 10^{-3} M$)

| *Addition of sodium bisulfite with* | *Sporulation* |
|---|---|
| Pyruvate ($10^{-2} M$) | — |
| Acetate ($10^{-2} M$) | — |
| α-Ketoglutarate ($10^{-2} M$) | — |
| Aspartate ($10^{-2} M$) | — |
| Malate ($10^{-2} M$) | — |
| Propionic acid ($10^{-2} M$) | — |
| Formic acid ($10^{-2} M$) | — |
| Citrate ($10^{-2}$) | + |
| *cis*-Aconitate ($10^{-2} M$) | + |
| Isocitrate ($10^{-2} M$) | + |
| Succinate ($10^{-2} M$) | + |
| Methylmalonic acid ($10^{-2} M$) | + |
| Malonic acid ($10^{-2} M$) | + |
| Glyoxylic acid ($10^{-2} M$) | + |

malate. The reversal by glyoxylic acid and ketoglutarate is to be expected, since the aldehyde and ketone groups would tie up the bisulfite and thus remove it from the sphere of action. The fact that malate does not reverse the inhibition of the bisulfite may indicate that bisulfite is also tying up the oxalacetate and thus breaking the cycle at that point.

The glyoxylic acid shunt may be needed for sporulation, but as yet, we do not have convincing proof. At the present time we are pursuing this investigation further with radioactive tracers, and hope, by this technique, to get conclusive proof or denial. Regardless of the cycle involved, all of the inhibitors we have studied are reversed by succinate; and succinate has proved to be the most effective reversing agent because it will reverse these inhibitors in smaller concentrations than any of the others. This leads us to suspect that succinic acid is an intermediate in the synthesis of spore material, and also perhaps, in the synthesis of DPA. There is some evidence against this

conclusion; in that, Martin and Foster[23], when they studied the incorporation of various types of labeled compounds into DPA, obtained little evidence for the incorporation of succinate. In their experiments there may have been an abundant supply of succinate within the cell, and therefore, it did not utilize succinate added from the outside. You may recall from data on the anaerobic culture, that we could separate the formation of the heat-sensitive spore from the synthesis of DPA and from the development of heat resistance. We have not been able to obtain such a separation in the case of the aerobes. We have, therefore, investigated other inhibitors to see if we could find some substance that would permit the synthesis of the spore structure but not the synthesis of DPA, and thus prevent the production of a heat-resistant spore. We have succeeded in obtaining this result with two inhibitors, ethyl oxamate and diethyl pimelate.

To pursue this study one needs some mechanism to differentiate between heat sensitive spores, vegetative cells, and germinated spores. In this case, it cannot be done by heating. Octyl alcohol proved to be suitable for this purpose. This alcohol is very toxic to vegetative cells, killing them almost instantly, and also will destroy germinated spores almost equally fast. Spores are extremely resistant to this chemical, and, as will be shown later, the heat sensitive spores are also resistant. Table IX shows the effect of octyl alcohol upon germinated spores and vegetative cells of *B. cereus*. Table X shows the effect of ethyl oxamate

TABLE IX

EFFECT OF OCTYL ALCOHOL ON THE VIABILITY OF SPORES, GERMINATED SPORES, AND VEGETATIVE CELLS OF *B. cereus* T.

| *Type of cells* | *Without octyl alcohol* | | *With octyl alcohol* |
|---|---|---|---|
| | *Viable* | *Heat stable* | *Viable* |
| Spores | $3 \times 10^8$ | $2.5 \times 10^8$ | $3 \times 10^8$ |
| Germinated spores | $1.6 \times 10^8$ | $10^5$ | $10^5$ |
| Vegetative cells | $6 \times 10^7$ | $<100$ | $<100$ |

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TABLE X

EFFECT OF TIME OF ADDITION ON ETHYL OXAMATE ($10^{-2}$ *M*) ON THE PRODUCTION OF HEAT-STABLE SPORES

| *Type of culture used* | *Octyl alcohols-stable cells/ml* | *Heat-stable cells/ml* |
|---|---|---|
| Spore inoculum | $2 \times 10^8$ | $6 \times 10^5$ |
| Active culture at 0 time | $4 \times 10^8$ | $8 \times 10^5$ |
| Active culture pH 5.2 (falling) | $7 \times 10^8$ | $1.5 \times 10^5$ |
| Active culture pH 5.8 (rising) | $6 \times 10^8$ | $6 \times 10^5$ |
| Active culture pH 7.1 (rising) | $1.5 \times 10^9$ | $1.4 \times 10^8$ |
| Active culture pH 7.9 (rising) | $8 \times 10^8$ | $2 \times 10^8$ |

upon the production of heat-resistant spores of *B. cereus*. It is to be noted from this that ethyl oxamate interferes with the formation of heat-resistant spores, whether it is added in the beginning to a spore inoculum or an active culture, or before or after the pH has started to rise. If one waits, however, until the pH has gone up to 7.1 or higher, it has no effect. Apparently, by this time, the synthesis of DPA has already progressed to the point where heat-resistant spores can be found. We have examined the inhibited cultures for DPA and find there are very small amounts present. It is to be noted that a few heat-resistant spores are formed, so that the ethyl oxamate does not block the synthesis of DPA completely; but it does interfere with the synthesis sufficiently, so that more than 95% of the spores that are formed are heat sensitive. The amount of DPA which is found in such preparations is slightly more than one would expect if one assumes that the heat-resistant spores have their normal content, and the heat-sensitive spores, none. It is possible, therefore, that some DPA may be present also in the heat sensitive spores.

Somewhat similar results are obtained with the diethyl pimelate. This inhibitor, however, interferes with the development of normal vegetative cells if it is added to the culture at 0 time, or very early in the growth of the vegetative cells. The vegetative

cells look abnormal, and, in fact, many of them lyse before they can begin to produce spores. This inhibitor may very well interfere with the synthesis of cell walls. If the inhibitor is added after the pH has started to rise (at which time the production of vegetative cells has been completed and presumably there is no further synthesis of cell wall), we find that the inhibitor does not interfere with the production of spores; but the spores which are produced are heat sensitive, as shown in Table XI. In fact,

TABLE XI

THE EFFECT OF TIME OF ADDITION OF DIETHYL PIMELATE (0.01 *M*) ON SPORULATION

| | *After 24-h incubation at 30° on shaker* | | | |
|---|---|---|---|---|
| *pH of culture at time of addition* | *Viable (cells/ml)* | *Octyl alcohol stable (cells/ml)* | *Heat stable (cells/ml)* | *pH* |
| 4.9 (falling) | <100 | <100 | <10 | 4.85 |
| 5.3 (rising) | $10^8$ | $1.3 \times 10^8$ | $5 \times 10^6$ | 5.4 |
| 6.3 (rising) | $1.3 \times 10^8$ | $1.7 \times 10^8$ | $2.5 \times 10^6$ | 5.45 |
| 7.3 (rising) | $1.3 \times 10^8$ | $1.5 \times 10^8$ | $1.5 \times 10^6$ | 5.4 |
| 7.8 (rising) | $4 \times 10^8$ | $2.5 \times 10^8$ | $1.5 \times 10^6$ | 5.45 |

For purposes of counting, cells were spun down and resuspended in 0.01 *M* phosphate buffer, pH 7.2.
Vegetative cells and germinated spores are killed immediately on exposure to octyl alcohol (0.06 ml/100 ml $H_2O$).

the results are almost identical with those obtained with ethyl oxamate. Here again, more than 95% of the spores are heat sensitive. These heat-sensitive spores appear to be perfectly normal, as far as staining is concerned. They are refractile like normal spores; they undergo germination with ordinary germination nutrients, as normal spores will; and they are resistant to octyl alcohol. They are extremely sensitive to heat, most of them being killed at 65° in less than 15 min. Their heat resistance is no greater than that in vegetative cells.

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TABLE XII

EFFECT OF DPA ON THE PRODUCTION OF HEAT SENSITIVE SPORES BY ETHYL OXAMATE OR DIETHYL PIMELATE

| *Addition* | *Time of addition* | *Octyl alcohol-stable cells/ml* | *Heat-stable cells/ml* |
|---|---|---|---|
| | | *Concn. of octyl alcohol 0.06 ml/100 ml water* | |
| None | | | $5 \times 10^8$ |
| Ethyl oxamate | 0 | $2.5 \times 10^8$ | $1.5 \times 10^7$ |
| Ethyl oxamate + DPA | 0 | $3.5 \times 10^8$ | $4 \times 10^8$ |
| Ethyl oxamate + DPA | 7 | $2.2 \times 10^8$ | $5 \times 10^8$ |
| Ethyl oxamate + DPA | 9 | $1 \times 10^8$ | $4.5 \times 10^8$ |
| Diethyl pimelate | 7 | $2 \times 10^7$ | $1 \times 10^5$ |
| Diethyl pimelate + DPA | 7 | $7 \times 10^7$ | $9 \times 10^7$ |

Both of these inhibitors can be reversed by dipicolinic acid added from the outside. The results are shown in Table XII. It can be observed from this that reversal can be obtained with dipicolinic acid when added from the outside, as much as seven to nine hours later. In the presence of DPA, the spores produced are heat resistant. A similar experiment cannot be made with diethyl pimelate because this substance cannot be added to the cultures at the beginning. But, if this is added to the culture at seven hours, we find that most of the spores are heat sensitive; whereas if we add dipicolinic acid at the same time, all of the spores are heat resistant.

To summarize our data, it indicates that the glyoxylic acid shunt is involved in the synthesis of spore material and dipicolinic acid. Some of the enzymes that are needed in this shunt appear not to be present in vegetative cells but are produced as adaptive enzymes after the sugar has been used up. Succinic acid appears to be important as an intermediate in the synthesis of the spore material, and perhaps also in the synthesis of dipicolinic acid. The synthesis of spore material and the production of a spore-like structure can occur, independent of the

synthesis of dipicolinic acid. The only function the dipicolinic acid plays in the process is to produce a structure that can protect the enzymes and make the spore heat resistant. Heat resistance cannot develop until after the DPA has been synthesized. This lends further circumstantial evidence to the theory that dipicolinic acid is involved in the formation of a complex which serves to protect the enzymes and makes them heat resistant.

ACKNOWLEDGEMENTS

The unpublished results reported herein should not be credited to any one individual but to a research team in which the following have been my coworkers: Dr. Krishnamurty Gollakota*, Research Associate and Assistant Professor of Research; Dr. Robert Collier** and Dr. Herbert Nakata***, former graduate students who have completed research for their theses on some phase of this problem: Mr. Ivan Goldberg and Mr. John DePinto, current graduate students; and Mr. Francis Engle, laboratory assistant, and Leena Narasimhan, research assistant. The above individuals are to be considered as co-authors.

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## DISCUSSION

Kindler: Foster has claimed that diaminopimelic acid is a precursor of dipicolinic acid. I wonder if diaminopimelic acid reversed the inhibition of $\alpha$-pimelic acid, and I also would like to ask if there is any evidence of conversion of dipicolinic acid into diaminopimelic acid upon germination?

Halvorson: The answer to your first question is that there is quite good evidence now that diaminopimelic acid is not a precursor of dipicolinic acid. Furthermore, it does not reverse the inhibition of $\alpha$-pimelic acid. Whether dipicolinic acid can be converted into diaminopimelic acid has not been tested.

LEES: I am afraid I am totally ignorant of these matters. Does the spore contain structural proteins or lipoproteins, and if so does this same mechanism confer stability on them? Secondly does this mechanism you postulated for controlling heat stability also confer resistance to lack of water in the case of the spore?

HALVORSON: These abnormal spores are resistant to chemicals like octyl alcohol. They are not heat resistant, and like normal spores, do not show enzymic activity. We found a virus infecting our spores at a very late stage of growth; it became incorporated in the spore particles, and these were heat resistant. We tried this with our particular spores, and found them to become infected in the same way, with partial protection against heat. There were few heat-resistant spores and these contained dipicolinic acid. It may be that dipicolinic acid is protecting the virus.

KEYNAN: What might be the mechanism of the protection given by dipicolinic acid to the spore?

HALVORSON: I wish I knew the answer to that. All we have so far is indirect evidence that a complex is involved, but the pesky complex breaks up when we rupture the spore so that our hands are tied and we need some new ideas before we can ask the right question and suggest the proper experiment.

KEYNAN: Will $\alpha$-dipicolinic acid inhibit sporulation in aerobic systems?

HALVORSON: It also does the same thing with anaerobic spores. Ethyl oxamate, indeed, prevents the formation of heat-susceptible spores in anaerobes.

KEYNAN: Does ethyl pyruvate interfere with the growth of the vegetative cell?

HALVORSON: This is the only one of the ethyl esters tested that had any effect on the growth of vegetative cells. However, it does not stop growth altogether. It also interferes with germination of spores.

KEYNAN: Does it interfere with sporulation or just with growth?

HALVORSON: With growth. Growth is reduced.

GROSSOWICZ: Is the dipicolinic acid found inside the spore or on its coat?

HALVORSON: I think Dr. Gerhardt of the University of Michigan claimed that it is inside the spore?

HALVORSON, JR.: Robinow made very thin sections and noticed that the cortex region between the outer spore wall and the inner membrane had about a third of the volume of the spore. It showed a well defined striated structure which disappeared on germination. It is suggested that the dipicolinic acid lies within this region. Gerhardt showed, however, that dipicolinic acid could be removed from the cell without injuring the cortex.

GROSSOWICZ: You mentioned a few inhibitors of some energy-yielding reaction inside the spore. Is there any evidence of necessity for protein synthesis in order that sporulation may take place?

HALVORSON: Joan Powell of Porton showed that there were special kinds of peptides when spores germinated, and I have assumed all along that these represent breakdown products of proteins, special proteins presumably. But we have not done any work on this, and I am not sure that the evidence is sufficient.

GROSSOWICZ: What is known about chemical transformations occurring during the ripening or the aging of spores?

HALVORSON: We have mostly speculations. The evidence found in our laboratory shows that L-alanine is found in aged spores, but not in the supernatant of fresh spores. Krask working at Camp Detrick found proteolytic enzymes present in the intact spores. These enzymes seemed to be heat resistant. This would be evidence that something happens during aging and perhaps the same during heat shock. I assume that there is a small breakdown of materials necessary for germination or alteration of the spore walls, so that things can get in more easily. We do know that with aging the germination requirements become simplified and the need for heat shock is reduced.

HESTRIN: Does addition of dipicolinic acid to the non-heat-resistant cells confer heat stability?

HALVORSON: Cultures to which ethyl oxamate has been added, and which normally should yield heat-sensitive spores, will produce heat-resistant spores on the addition of dipicolinic acid.

# STUDIES ON THE GERMINATION OF SPORES OF *BACILLUS LICHENIFORMIS*

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Many of us will agree with Orin Halvorson's statement in a recent review that the 'trigger mechanism for germination is one of the most interesting and unique mechanisms found in nature'[1]. Although much research and speculation has been devoted recently to this process and although a picture is beginning to emerge of the events leading to the germination of bacterial spores, the exact mechanism of the latter has not yet been elucidated. Many of the facts connected with the 'trigger reaction' leading to germination have been described in the preceding lectures. I will, therefore, repeat only briefly some of those on which our conception of the nature of induction of germination is based.

It is well known that freshly grown bacterial spores of many species will not germinate readily after harvesting. In order to induce germination in these spores they have to be 'aged' or 'heat activated'. There is much evidence today that during 'aging' or 'heat treatment' some dormant enzyme system is activated. Adopting Harlyn Halvorson's conception, one might say, that the 'heat treatment' breaks down a mechanism which controls dormancy.

'Aged' or 'heat-treated' spores may be triggered readily and converted into heat-sensitive germinated cells by the action of a number of substances, among which L-alanine is the most common. The mechanism of the action of alanine in this process is not known, but both substrate and catalytic activity have been suggested[2]. O'Connor and Halvorson[3] have shown that germination is attended by deamination of both endogenous and exogenous alanine by an L-alanine dehydrogenase to yield pyruvate. Much evidence has accumulated that metabolism of pyruvate is involved in germination. Inhibitors of pyruvate

metabolism prevent the germination—at least in most species of bacteria tested. These reactions have been shown to be part of the metabolism of the germinating spore, but it is not yet possible to define which of them is responsible for the *beginning* of germination and therefore constitutes the 'prime event'.

It might be helpful in the study of this problem to find a system in which it would be possible to separate the 'prime event' ('trigger reaction') from later occurring metabolic steps. Working with a strain of *B. licheniformis* we found that 'triggering', *i.e.* initiation of germination, can be brought about under conditions which are distinct from those under which germination of a 'triggered' cell can occur. Spores of this strain could be 'triggered' by L-alanine at temperatures above 20°, and after exposure to such temperatures for a short time were able to germinate (as manifested by a drop in optical density) even at a temperature as low as 0°. We interpret this to mean that the 'trigger reaction' can occur at a temperature higher than is required for a subsequent metabolic step. Utilising this observation, and with the intention of investigating the initiation of germination separately from its subsequent steps, an experiment was designed in which the heat-activated spores were preincubated with L-alanine at 37° for a few minutes, and were then cooled to 15° or 18°.

As previously stated, alanine failed to initiate activation in cells treated directly at 15°. Nevertheless, optical density of spore suspensions preincubated at 37° for a few minutes continued to decrease at 15°. The final drop in optical density at 15° and 18° depended on the duration and temperature of preincubation.

Fig. 1 presents data of such an experiment. Two minutes of preincubation of a spore suspension at 37° are not enough for germination to start after transfer to 0°, but after 4 min of preincubation, about 30% of the spores are activated. This can be seen when the preincubated suspension is transferred to 0°.

The 'prime event' starting the chain of reactions apparently precedes in time the measurable manifestation of germination.

*References p. 70*

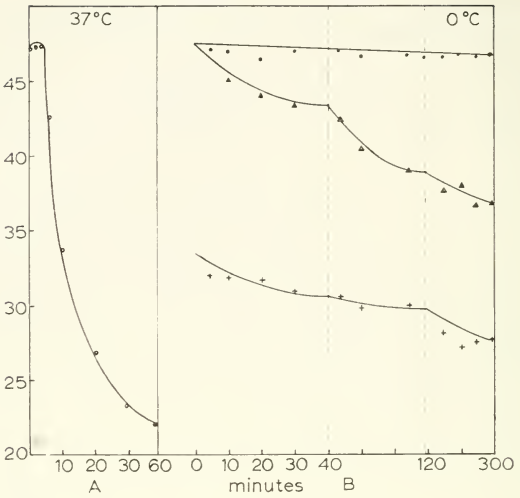


Fig. 1. Decrease in optical density of a spore suspension of *B. licheniformis* at 0° after preincubation with alanine at 37° for different times.
Conditions of experiment: Spores were washed 30 times, heat shocked for 16 h at 60° in water, and suspended in 7 × $10^{-2}$ *M* phosphate buffer, pH 8. Activation by L-alanine in final concentration = 1/75 *M*. Total volume 1.5 ml in test tubes of 6 mm diameter. Measurement of optical density in Coleman Junior spectrophometer at 540 $\mu$. A, Decrease in optical density of spore suspension incubated at 37°. B, Decrease in optical density of spore suspension incubated at 0° after preincubation at 37°. ●, preincubated for 0–2 min; ▲, preincubated for 4 min; +, preincubated for 10 min.

This can be shown from the preceding observations. In the above experiment a drop in optical density started at 37° only after 6 min. However, when spores which did not yet show a decrease in optical density after preincubation for 4 min were transferred to 0°, they showed germination. This means that the 'trigger reaction' occurred some time between the second and the fourth minute, preceding any other measurable change. The fact that the induction of germination precedes any measurable change has been established previously by Harrell and Hal-

vorson[2], who demonstrated the existence of a time interval between a very short exposure to L-alanine and the subsequent manifestation of germination, and also by Woese[4], who showed the existence of a time interval between alanine activation and the subsequent release of dipicolinic acid.

All the evidence that may be derived from our experiment of 'double temperature' exposure shows that whatever occurs at temperatures below 20° depends entirely on preincubation at the higher temperature. As already stated, extent of germination, as measured by total drop in optical density at 15° or 18°, depends on duration and temperature of preincubation at 37°. The temperature dependence is demonstrated in Table I, which

TABLE I

INFLUENCE OF TEMPERATURE OF PREINCUBATION ON THE EXTENT OF GERMINATION AT 18° OF SPORE SUSPENSIONS OF *B. licheniformis*
(Conditions as in experiment presented in Fig. 1)

| *Temperature of preincubation for 6 min* | *Per cent of decrease in optical density after 6 min of preincubation** | *Final drop in optical density after transfer to 18°*** (%) |
|---|---|---|
| 24° | 0 | 5 |
| 29° | 2 | 20 |
| 36° | 19 | 38 |

* Per cent of decrease in optical density expressed as:

$$\frac{\text{initial optical density} - \text{final optical density}}{\text{initial optical density}} \times 100.$$

** The drop in optical density was considered as final when no measurable change occurred during 1 hour.

gives data of an experiment in which spore suspensions were exposed for 6 min to different temperatures above 20° and then transferred to 18°.

If we suppose that this procedure separates events occurring during germination, we have to conclude that whatever occurs at the lower temperature is an outcome, but not a part of the

*References p. 70*

'trigger reaction' itself. Therefore, it would be interesting to compare the action of inhibitors on the 'alanine activation' during preincubation at 37° with that of the reaction occurring at 15°, with the idea in mind that inhibitors which prevent germination during preincubation at 37° but have no influence on events occurring at 15° are true inhibitors of the primary 'trigger reaction', while those preventing germination at 15° are inhibitors of other metabolic reactions subsequent to the primary triggering.

Among inhibitors known to prevent L-alanine activation, D-alanine is active only if added before, together or immediately after addition of L-alanine. In our 'double temperature' experiment it was demonstrated that D-alanine prevented germination when added during preincubation at 37°, in the first stage of the experiment, but did not influence events at 15° when added at the second stage.

Various salts inhibited germination when added during preincubation. When, however, these salts were added to spore suspensions after preincubation at 37° during the second stage of the experiment (at 15°), they were without influence on the rate or extent of germination. $ED_{50}$ of KCl, $NaNO_3$ or LiCl when added together with L-alanine was found to be $8 \times 10^{-2}$ *M*. Some other aspects of the salt inhibition of L-alanine activation may also be of interest. Salts of divalent cations were more active than monovalent ones: $ED_{50}$ values were $5 \times 10^{-3}$ *M* for $MgCl_2$ and $6 \times 10^{-4}$ *M* for $CaCl_2$. The inhibiting action of the salts was initially reversible, since it was abolished when the cells were washed 10–15 min after salt addition. When, however, spores were exposed to the salts for as long as 2 h the germination-inhibiting action could no longer be reversed by washing in a centrifuge. Inhibition by NaCl decreased with increasing amounts of L-alanine. The salt inhibition was therefore competitive in respect to L-alanine.

Other inhibitors, *e.g.* $HgCl_2$ and octyl alcohol prevented germination at whatever stage they were added, whether at 18° or at 37°. Presumably they block some metabolic

reaction in germination other than the primary reaction.

The following experiment was carried out in order to learn whether octyl alcohol, 0.01 *M* acts on the 'trigger reaction' itself in addition to its known action on subsequent metabolic steps. Spores exposed to L-alanine for 10 min at 37°, with and without the inhibitor, were washed 5 times in the cold, in order to remove both the alanine and the inhibitor. After washing, the spores were resuspended, incubated and the decrease in optical density measured. Those exposed to alanine alone germinated readily after resuspension and incubation, while those preincubated in L-alanine and octyl alcohol did not germinate (Spore counts showed that the octyl alcohol did not kill any of the spores). This seems to indicate that octyl alcohol is inhibitory both at the 'trigger reaction' and at subsequent metabolic steps. Octyl alcohol is known to inhibit L-amino-oxidases; therefore the above observation supports the idea that an L-amino-oxidase might be concerned in the triggering of germination. In a similar experiment, ethyl pyruvate has been shown to inhibit L-alanine activation during preincubation at 37° for 10 min. This appears to confirm the hypothesis that pyruvate metabolism is an integral part of the primary event leading towards germination.

It should be noted that temperature is not the only device by which the 'trigger reaction' can be separated from subsequent metabolic steps in this strain. Another means has been provided by our finding that whereas L-alanine fails to activate spores at pH below 6.5 the spores are able to germinate in the range pH 5.0 to 6.5 provided that they have been triggered previously by L-alanine at the higher pH.

I would like finally to raise a point of nomenclature. In this paper several designations have been applied to the process of initiation of germination, namely 'trigger reaction', 'first step', 'prime event', 'alanine activation', and others. Might it not be useful to have one generally agreed designation for this process?

In summing up, we are able to say that a prime event in the germination reaction has been resolved by our experiment from

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some subsequent steps involved in germination. The system proposed might be useful in the analysis of the sequence of biochemical events during the germination of bacterial spores.

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## DISCUSSION

GROSSOWICZ: Does the experiment with octyl alcohol mean that, after what you call the 'prime event' an induction of enzyme synthesis takes place?

KEYNAN: There is no evidence of enzyme induction, there is of course room for speculation.

HARPAZ: Is anything known on the effect of mercury compounds in very small doses on the stimulation of germination?

O. HALVORSON: At the concentrations we have used, the mercury compounds inhibited germination.

KEYNAN: We tested mercury compounds and they always stopped germination the minute they were introduced into the system. Whether it affects the triggering, I do not know, but it certainly stops the second step.

HESTRIN: I do not quite understand the implication of the fact that only part of the spore population germinates depending on the temperature of pretreatment.

KEYNAN: Some spores require lower temperatures and shorter times, others higher temperatures for longer times.

O. HALVORSON: I agree that individual spores have varying requirements for germination.

# THE BIOCHEMICAL NATURE OF THE DORMANT STATE IN THE BACTERIAL ENDOSPORE*

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The biochemical nature of the dormant state of bacterial endospores might seem at first to be a contradictory statement since the dormant state is generally associated with the absence of metabolic activity. This situation is particularly true of the bacterial endospore which is probably the most nearly inert biological system known. A biochemical description of the dormant state must include an analysis of those chemicals whose inclusion is essential to the production and maintenance of dormancy as well as the enzymic reactions available for triggering the breaking of dormancy.

## THE CHEMICAL NATURE OF THE DORMANT STATE

A number of quantitative chemical differences have been observed between spores and vegetative cells[1]. The spores have lower levels of D-amino acids, free amino acids, lipid, polysaccharide, RNA and water whereas they have higher levels of total nitrogen, protein-bound phosphorus and labile protein-bound phosphorus. Our knowledge of the water content of spores is unsatisfactory. Henry and Friedman[2] reported that *B. megatherium* spores contained 58% water whereas the vegetative cells contained 80% water based on weight after drying. Ross and Billing[3], employing refractive index measurements, observed values for spores comparable with those of dehydrated proteins suggesting that spores are essentially

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dehydrated. Murrell and Scott[4] arrived at similar conclusions in considering the heat resistance of bacterial spores at various water activities. These same workers, however, reported that 99% of the volume of the spore was exchangeable with deuterium-labeled water[5].

The more interesting chemical differences are those which can be correlated with the degree of dormancy. The first of these was demonstrated by Curran *et al.*[6] who found a high level of calcium (5%) in bacterial spores compared to 0.54% in vegetative cells. Sporulation in low calcium medium resulted in heat-sensitive spores. These findings have been confirmed by others[7–11]. Slightly higher levels of other bivalent cations, $Fe^{2+}$, $Ni^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Al^{3+}$, are found in spores than in vegetative

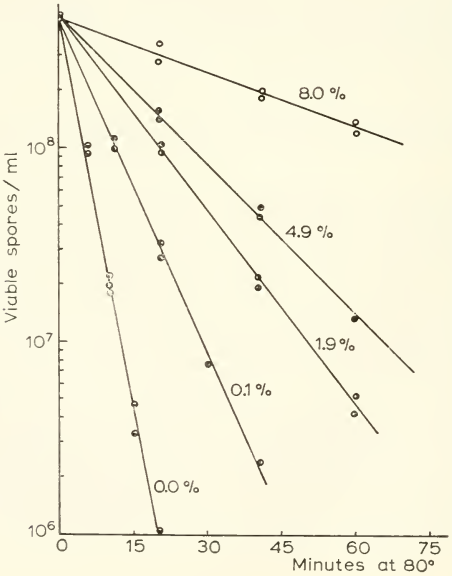


Fig. 1. Kinetics of heat inactivation of *B. cereus* strain T spores containing various levels of dipicolinic acid (DPA). The numbers refer to the per cent dry weight of DPA.

cells, some of which can substitute for $Ca^{2+}$ in producing heat-resistant forms[10].

The second correlation followed from the discovery that spores contain massive quantities of a chelating agent, dipicolinic acid (DPA), which is absent in vegetative cells[12]. This unique compound, which is found just prior to or coincidental with heat resistance, has attracted considerable interest. The DPA level varies from 5–15% depending on the species under study. The discovery several years ago that the DPA content could be varied by changes in the growth supplements of the medium[13] provided an opportunity for relating this to heat resistance. The rate of heat inactivation of some of these spores is shown in Fig. 1. Single heat inactivation curves were observed with considerable variations in heat resistance. The correlation of their dipicolinic acid content with viability and heat resistance is shown in Fig. 2. Above 1% DPA, the spores are viable and their heat resistance is proportional to DPA content. Below

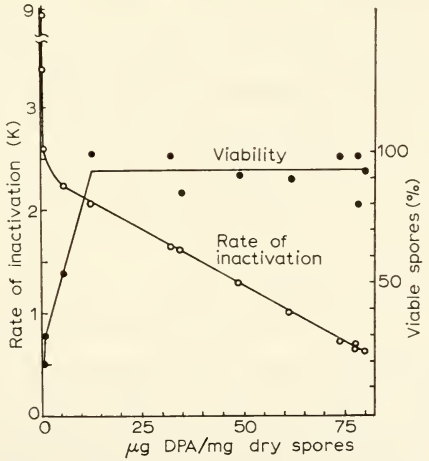


Fig. 2. Effect of the dipicolinic acid content on the viability and heat resistance of *B. cereus* strain T spores. The rate of inactivation was measured at 80°.

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1% DPA, viability decreases and a higher dependence of heat resistance on DPA is also evident. Black, Hashimoto and Gerhardt[7], employing the technique of endotropic sporulation in distilled water, produced low DPA spores which were found to be relatively susceptible to heat. This has recently been extended to show a correlation between the $Ca^{2+}$ content of the sporulation medium and DPA biosynthesis[14]. The presence of these two may be manditorily coupled such that $Ca^{2+}$ is required for DPA synthesis and the latter as a means of chelating and maintaining high levels of $Ca^{2+}$ or other divalent cations.

## RESPIRATORY ACTIVITY OF DORMANT SPORES

The second biochemical description of spores concerns their apparent physiological inertness. Consider, for example, the aerobic sporeformers which require oxidative reactions for the supply of both energy and building materials. The respiratory rate ($Q_{O_2}$) on glucose for vegetative cells ranges from 60–100. Reports on spores have varied considerably. Levinson and Hyatt[15] reported a $Q_{O_2}$ value of 2.4 in a preparation of *B. megatherium* spores containing approximately 10% germinated forms. Crook[16], in examining a well washed suspension of *B. subtilis* spores, observed a $Q_{O_2}$ value of 0.3 by means of a microrespirometer. Unfortunately he did not report the percentage of germinated forms. In any such studies the cleanliness of the spores and the absence of germinated forms are essential to measurements of the metabolic activity of the dormant forms themselves.

We have reexamined this point by extensively washing fresh spores of *B. cereus* 12–16 times until they are free of detectible levels of germinated forms[17]. When dormant spores were tested at very high densities (30 mg/Warburg cup) there was no detectible $O_2$ uptake after 60 min in the presence of glucose, whereas heat-shocked spores took up 60 $\mu$l $O_2$ and aged heat-shocked spores 300 $\mu$l $O_2$. In the latter case extensive germination is associated with respiratory activity. Based on the limitations of the manometric technique employed, the $Q_{O_2}$ of the

dormant spores is less than 0.05 or about $\frac{1}{2{,}000}$ of the activity of corresponding vegetative cells.

These findings, which have been observed by a number of workers, illustrate several features of dormant endospores:

1. Their overall respiratory activity is negligible if not completely inactive;
2. Respiratory activity is acquired prior to germination by appropriate activation either by heat or by chemicals;
3. Germination leads to more full metabolic activity. In this latter case, Levinson and Hyatt[15] have shown that this can be largely achieved under nutritional conditions where protein biosynthesis is negligible. It is therefore clear that the metabolic systems involved are pre-existent in the dormant spore.

## ENZYME PATTERN OF DORMANT SPORES

It was originally believed that dormant spores were devoid of enzymic activity. This is clearly not the case. Since the discovery of alanine racemase[18] and catalase[19, 20], an increasing number of enzymic activities have been recognized in dormant spores. In some cases, such as pyrophosphatase[21] and alanine racemase[18], the enzyme content is higher in spores than in vegetative cells. A similar situation exists regarding enzymic reactions demonstrable in extracts of dormant spores. During the past seven years there has been an exponential increase in the number of enzymes recognizable in spores—suggesting that the enzyme pattern of spores may closely resemble that of the sporulating cell.

Three rather broad classes of enzymes can be recognized in spores:

(a) Enzymes active in intact dormant spores;
(b) Enzymes dormant in intact spores, but recognizable following activation;
(c) Enzymes active only in extracts of dormant spores.

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SIGNIFICANCE OF THE ENZYMIC ACTIVITIES OF DORMANT SPORES

It is easy to imagine that the establishing of a metabolically inert dormant state can be of selective advantage to an organism. However, one would also expect that selective pressures would be operative in favor of those which can survive dormancy and rapidly revert to vegetative forms in which the full complement of metabolic activity is unmasked and active. The microbiological literature, especially the applied aspects, are rich in examples of delayed dormancy. For example, McCoy and Hastings[22], employing single-cell technique, found that in *Clostridium acetobutylicum* the germination of 5% of the freshly harvested spores was delayed from 11 to 117 days and one spore, isolated from a year-old culture, required 222 days to germinate. The question is raised in these examples of the physiological defect in these spores displaying delayed dormancy.

*A priori* one can visualize two types of metabolic activity which can be recognized in spores:

(1) The maintenance of a low level of metabolism for maintenance of the dormant state;
(2) The maintenance of enzymes essential for the triggering of germination of activated spores and unmasking of overall metabolism.

The question of whether or not metabolism is required, or even present, in dormant spores has unfortunately not been sufficiently considered. This problem should be subject to test in either dormant or delayed dormant spores. Germinated or even activated spores differ sufficiently in density that it is possible to separate clean dormant spores by density gradient sedimentation as material for examination. If there were any appreciable metabolic activity present in these spores one should be able, for example, to demonstrate the incorporation of $^{32}P$ phosphate into ATP. Employing carrier-free $^{32}PO_4$, and carrier isolation, the sensitivity of the respiratory activity should be amplified by

over a million fold. Such studies would be very interesting with regard to spores of varying states of dormancy.

The second type of metabolic activity associated with the triggering of germination in activated spores is more readily subject to experimental analysis and has been in large our own approach to the problem. There are essentially two stages involved: (a) an activation reaction which may be reversible and (b) triggered germination of activated spores.

## ACTIVATION

Activation serves to start the biological clock in germination which under suitable conditions can lead to the loss of heat resistance within a 10-min period. Activation can be achieved in a number of ways: heat shock, chemical agents, storage or mechanical means. The recognizable events of activation are the unmasking of a number of enzyme systems, the loss of some spore components, including DPA, disruption of the integrity of the exosporium and probably an increased permeability.

Our knowledge of the sequence of events and their relative importance towards poising the system for germination is incomplete. It is not clear whether or not the process is purely physical or may be enzymic. Clearly more work is required in this direction. The activation of the lysozyme-like lytic enzyme, which is active against exosporium and spore walls, might provide an explanation. In *B. megatherium*[23], $Mn^{2+}$-initiated germination can be understood by the activation of enzyme activity by $Mn^{2+}$ and the subsequent liberation of endogenous germinating agents, *e.g.* L-alanine.

## TRIGGERING REACTIONS IN GERMINATION

The success of germination is undoubtedly dependent upon the physiological competence of the activated spore. The enzymic nature of germination can be inferred from a number of considerations:

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1. Germinating agents are usually normal metabolites and in a number of cases disappear during germination;
2. Stereospecific binding sites can be recognized for germinating agents which are subject to competitive inhibition;
3. The temperature dependence of germination is that expected of an enzymic reaction[24, 25];
4. Germination can be blocked by a number of metabolic poisons.

The primary objective is the recognition of the primary reaction and also the metabolic reactions essential to germination.

We have approached this problem by characterizing the germinating agents of *B. cereus*[1, 26]. The common feature of the germination stimulants appears to be their biochemical relationships rather than their structural relationships. Products of hexose metabolism, pyruvate and its normal degradation products, can act as germinating agents. These findings have led us to examine the metabolism of germinating agents by activated

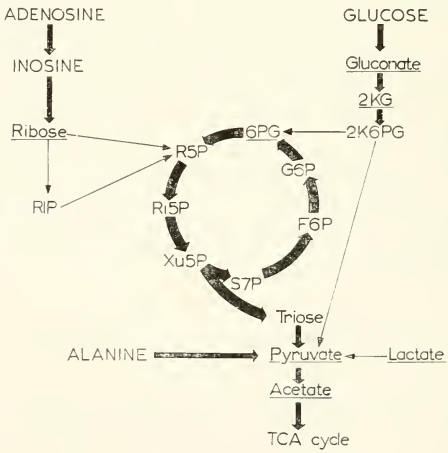


Fig. 3. The pathway of glucose oxidation in *B. cereus* strain T spores.

spores and extracts of activated spores. A summary of some of the individual reactions demonstrated in extracts by enzyme purification and end product analysis are shown in Fig. 3, ref.[27]. The compounds in large letters are primary germinating agents and those underlined are less effective germinating agents. Glucose is initially oxidized to gluconate by a soluble DPN-linked glucose dehydrogenase. Spores are devoid of hexokinase, phosphoglucomutase, phosphohexokinase and aldolase and subsequently lack a functional glycolytic system. Gluconate is converted to 2KG by a TPN-linked system which is in turn phosphorylated to 2K6PG by an ATP-requiring 2KG kinase. 2K6PG is reduced in part to 6PG by a DPNH requiring 2K6PG reductase and in part to pyruvate by an as yet unidentified pathway. Spore extracts contain a complete functional hexose monophosphate shunt which leads to triose formation which is in turn converted to pyruvate. Pyruvate is oxidatively decarboxylated to acetate which is then oxidized to $CO_2$ by a particulate tricarboxylic acid cycle.

The observation that inosine serves as a more effective germinating agent than adenosine led to the discovery of an adenosine deaminase which converts adenosine to inosine and a heat-stable hydrolytic nucleoside ribosidase which cleaves inosine to the free base and ribose[28, 29]. Krask and Fulk[30] have demonstrated in these extracts the presence of a $Mg^{2+}$ activated ribokinase which in the presence of ATP converts ribose to R5P. Alternatively they found that some of the ribose is converted to R1P from adenosine by nucleoside phosphorylase which is converted to R5P by an active phosphoribomutase.

The cardinal role of L-alanine in the germination of aerobic spores has led to a further search for its metabolism. The observation that L-alanine is consumed during germination[20, 26, 31, 32] led to the demonstration that isotopically labeled L-alanine is converted to pyruvate and $NH_3$[33]. The relevant enzyme, alanine dehydrogenase, has since been isolated and characterized[34].

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### PYRUVATE HYPOTHESIS

The hypothesis that the germination agents are metabolized to a common intermediate which is responsible for germination, is supported by the above findings that pyruvate may be derived from alanine, adenosine or glucose. If germination requires the formation of products of pyruvate oxidation, one would expect that precursors of pyruvate would support a germination which was sensitive to inhibitors of pyruvate oxidation, whereas products of pyruvate oxidation would permit germination which was insensitive to these inhibitors. An example of this was observed for spores of *B. cereus*[26]. Germination which normally occurs in the presence of glucose, pyruvate, 6PG, R5P or 2KG was inhibited by hexetidine, an inhibitor of pyruvate oxidation. The inhibition was reversed by cocarboxylase. In the presence of hexetidine, pyruvate and $NH_3$ accumulate. Germination in the presence of acetate, however, was insensitive to hexetidine. Similar results have been obtained with arsenite[33]. Recently Church[35] has found that intermediates of the tricarboxylic acid cycle, fumarate, succinate, citric, and *cis*-aconitic acid will initiate germination. One might postulate, for example, that germination requires energy, the formation of $\alpha$-keto acids for amino acid synthesis or of organic acids which act as sequestering agents with the heavy metals present in spores. Although a further clarification of this will require further experimentation, it is clear that germination involves the initial mediation of energy-yielding reactions in a system characterized by a burst of degradative reactions.

### THE ELECTRON TRANSPORT SYSTEM AND ITS REGULATION BY DPA

The pyruvate hypothesis, which we have just outlined, places also an increasing dependence of germination on the activation and functioning of the electron transport system of the spores. Since this system is essentially absent in the dormant spore, its activation is essential to oxidative reactions. Although one might *a priori* invoke a number of hypotheses to explain the inactive

state of these enzymes, the approach to the problem is largely guided by experimental opportunities. This was provided by Harrell and Mantini[36] by the finding that both the glucose oxidizing capacity as well as the release of DPA was proportional to the length of the heat shock period. These concurrent activations suggested that the two phenomena were closely related. It was thought that during heat activation perhaps an enzyme inhibitor was removed or an enzyme stimulator released which would affect the activity of the overall respiratory system.

Together with Harrell[37, 38] we observed in extracts of heat-activated spores that the oxidation of glucose or of DPNH could be stimulated three fold by the addition of DPA, as shown in Fig. 4. DPA was not metabolized nor was the activity of the

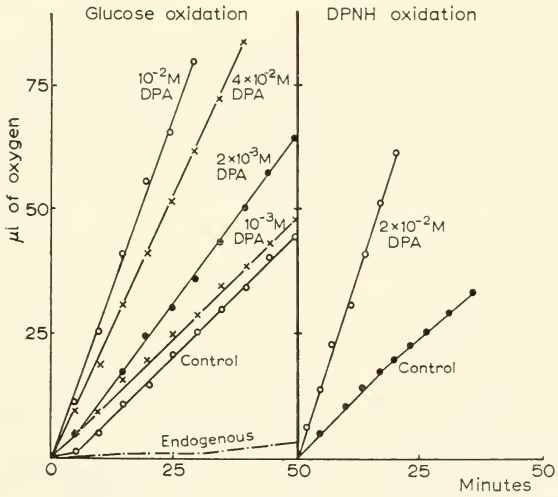


Fig. 4. Stimulation of glucose and DPNH oxidation by dipicolinic acid. The Warburg vessels contained: glycylglycine buffer, pH 7.3, 75 μmoles; enzyme fraction with 4 mg protein; DPA as indicated and (a) glucose, 20 μmoles; DPN, 0.7 μmoles; (b) DPNH, 10 μmoles. Final volume, 1.8 ml. Center well contained 0.2 ml of 20% KOH. Incubation temperature, 30°.

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first enzyme active on glucose, the DPN-linked glucose dehydrogenase, stimulated by DPA. It was apparent from these findings that DPA was acting in stimulating the electron transport system rather than at the level of substrate oxidation. Since DPA is a powerful chelating agent and heavy metals have been implicated in controlling electron transport in other systems, it seemed not unreasonable that it may be acting here by virtue of its chelating potential. Such mechanism of action was in fact suggested by an analysis of the DPA stimulation of the ATPase of spores[37]. The ATPase was slightly inhibited by $Mn^{2+}$ and perhaps certain other divalent metals present in the spore extract. An experiment designed to test the chelation hypothesis with the soluble DPNH oxidizing system was negative[37]. The stimulation could not be attributed to the removal of an inhibitory metal since two other chelating agents, 8-hydroxyquinoline and versene did not stimulate the enzyme, and prior dialysis against DPA did not abolish the DPA effect.

A clearer understanding of the mechanism of DPA stimulation has been handicapped by our knowledge of the electron transport system operative in spores. A number of observations have made it clear that it differs in several respects from that of the vegetative cells. Keilin and Hartree[23] reported that spores had less than 6% of the cytochromes present in vegetative cells. Hachisuka *et al.*[49] observed similar results and also found that overall germination is characterized by a development in the respiratory system. Spencer and Powell[39], on the other hand, showed that the flavin content does not vary during germination. Nakada *et al.*[40] observed that spores are less sensitive to cyanide than are vegetative cells and that germination is accompanied by cytochrome synthesis.

These observations led us to a closer examination of the enzymes normally associated with electron transport. A comparison of some of these in extracts of vegetative cells and in extracts of activated spores[8] is shown in Table I. In vegetative cells DPNH oxidation is primarily associated with a particulate system rich in DPNH-oxidizing enzymes and cytochromes.

TABLE I

A COMPARISON OF ELECTRON TRANSPORT ENZYMES IN *B. cereus* CELLS AND SPORES

| *Enzyme* | *Source* | |
|---|---|---|
| | *Vegetative cell spec. act.** | *Spore spec. act.** |
| Particulate DPNH oxidase | 24.9 | 0.09 |
| Soluble DPNH oxidase | 0.06 | 0.30 |
| DPNH cytochrome *c* reductase | 3.9 | 0.24 |
| Diaphorase | 7.56 | 5.88 |
| Succinic cytochrome *c* reductase** | 0.36 | 0 |

* $\mu$Moles of DPNH oxidized/h/mg of protein.
** $\mu$Moles of succinate oxidized/h/mg of protein.

Spores on the other hand have little particulate activity but have a higher content of soluble DPNH oxidase. These findings as well as the cyanide-sensitive and cytochrome assays suggest the following electron transport systems of the two organisms (Fig. 5).

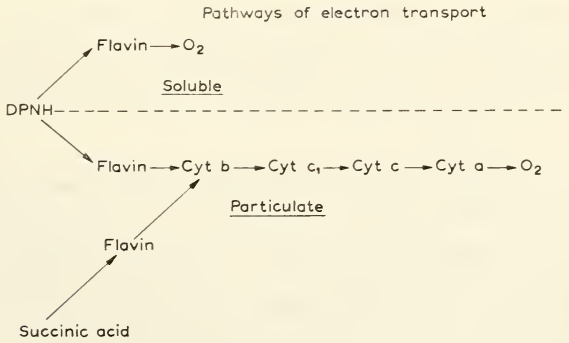


Fig. 5. The soluble and particulate electron transport system of spores and vegetative cells.

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We have recently purified a number of these enzymes present in spores[8]. Of particular interest was the DPNH oxidase which is the primary route of DPNH oxidation. In Warburg studies with a 20-fold purified enzyme, DPA stimulated oxygen uptake 3 fold while FMN stimulated oxygen uptake 9.4 fold. The lack of inhibition of the enzyme by cytochrome inhibitors, as well as the spectrum of the enzyme, suggest a flavoprotein oxidase employing FMN as a cofactor.

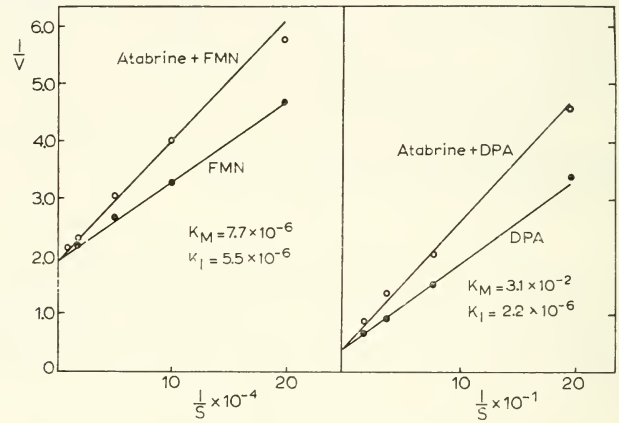


Fig. 6. Competitive inhibition of FMN and DPA by atabrine on the soluble DPNH oxidase. Reaction mixtures contained 0.1 *M* phosphate buffer, pH 7.3, $5 \times 10^{-6}$ *M* atabrine, $1 \times 10^{-4}$ *M* DPNH, enzyme, plus FMN and DPA as indicated. Optical density changes were followed at 340 m$\mu$ at 25°.

DPA and FMN appear to compete for the same site (Fig. 6). Atabrine, a flavine analog, competitively inhibits the stimulation of DPNH oxidation by either DPA or FMN. The affinity constant for atabrine is essentially the same calculated from both systems. DPA depresses the rate of FMN stimulation, this inhibition being reversed by higher concentrations of FMN.

DPA thus not only can substitute for FMN in stimulating the enzyme but also competes with FMN for the enzyme. This raises the interesting speculation that DPA, which has the

pyridine ring structure in common with DPN, may act as a cofactor. If it were, one would expect that an enzyme-bound reduced form of DPA was formed. An intermediate of this type, dihydrodipicolinic acid, has recently been postulated by Powell and Strange[50] who suggested the following mechanism for the synthesis of DPA from diketopimelic acid (Fig. 7).

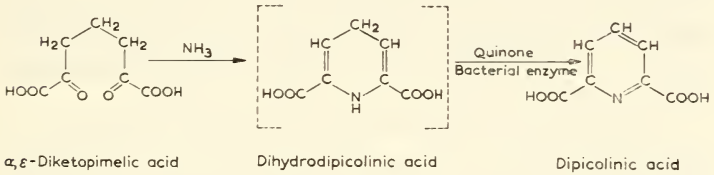


Fig. 7. Pathway of dipicolinic acid synthesis.

Assuming that DPA can act as an electron acceptor it provides an explanation for a number of phenomena associated with dormancy:

(1) Burst in respiratory activity of sporulation associated with DPA synthesis[41];
(2) Anaerobic germination where it may act as an electron sink substituting for oxygen[42];
(3) Rise in respiration following activation and germination[36] by stimulating the soluble oxidase pathway[27, 37].

## THE MECHANISM OF L-ALANINE UTILIZATION

In the light of the previous discussion it is evident that the DPA-accelerated oxidation of pyruvate or products of pyruvate is essential to germination. The prominent role played by L-alanine can be understood since it represents one of the most direct precursors of pyruvate among the germinating agents. The primary event in the interaction of L-alanine with activated spores has thus far remained obscure. In principle, this could be

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recognized by extrapolating the metabolism of alanine to time zero. The collective literature on spores suggests that a specific receptor site exists on the spore which, following combination with L-alanine, leads eventually to germination. This view led us over 6 years ago to start a search for the identity of the L-alanine binding site on spores of *B. cereus*. The discovery of an active alanine racemase[18] suggested this as the active site.

This was ruled out, however, since not only is L-alanine dependency on germination of various strains independent of alanine racemase activity, but also spore germination proceeds under conditions in which the enzyme is inactive[43].

The amount of L-alanine required to initiate germination is small. When spores are exposed for 45 seconds to $^{14}$C-L-alanine the binding of 200 molecules of L-alanine is sufficient to enable 40% germination[44]. Later it was observed[26, 31] that $NH_3$ and pyruvate release followed L-alanine disappearance. The rate of $NH_3$ release from L-alanine is dependent on heat activation while the amount of $NH_3$ and pyruvate formed, when pyruvate oxidation is blocked by arsenite, is greater than that expected from the L-alanine added[33].

In order to estimate the endogenous contribution to the release of $NH_3$, the recovery of labeled $NH_3$ from $^{15}$N-L-alanine was followed[33]. After 10 min incubation with spores, over 90% of the $NH_3$ was derived from endogenous sources. Employing $^{14}$C-labeled alanine and an arsenite block for pyruvate, 89% of the pyruvate recovered was derived from endogenous sources. The equimolar pyruvate and $NH_3$ recoveries suggested that compounds identical to or closely related to alanine are released. This was further confirmed by reisolation of the alanine from the medium in the absence of arsenite and demonstrating a 50% decrease in the specific activity of the exogenous alanine. Such dilutions are not unexpected since from the work of Powell and Strange[45], activation and germination are accompanied by a depolymerization of the outer layers of spores which are rich in alanine.

From the isotope studies the conversion of L-alanine to

pyruvate and $NH_3$ is clearly established. If the deamination of exogenous alanine is an initial step in germination, then the preferential metabolism of exogenous alanine would be expected in the early stages of germination. This was confirmed by observing the liberation of $^{14}CO_2$ from $C_1$ labeled alanine[33]. The highest specific activity was observed initially (after only 10 min incubation—the earliest point at wich sufficient $CO_2$ is produced to permit isolation). The findings are at least consistent with the view that the deamination of exogenous L-alanine precedes that of endogenous alanine and may be an initial step.

## NATURE OF THE INITIAL BINDING SITE FOR L-ALANINE

The above findings suggest that the L-alanine binding site is the deaminating enzyme or an earlier step. If it is the enzyme itself, the specificity of the alanine binding site on the spore should be identical to that of the deaminating enzyme. To provide information for this comparison we have purified the relevant enzyme over 60 fold from activated spores and characterized it[34]. The purified enzyme, an L-alanine dehydrogenase, carries out a DPN-linked deamination of L-alanine to pyruvate and ammonia. The reaction is reversible, specific for DPN, and has an activation energy of 8,200 cal/mole. The affinity constants for substrates and products as well as the other collective data on its properties identify the enzyme as the same as that reported in *Bacillus subtilis* vegetative cells by Piérard and Wiame[46], in *B. cereus* vegetative cells by ourselves[34] and in *Mycobacterium* by Goldman[47].

The amination reaction requires a proton which accounts for the fact that the reverse reaction is favored by more acid conditions. The pH optimum for deamination is high, about pH 10, which is in agreement with the ability of spores to germinate at high pH.

If the L-alanine dehydrogenase is the initial step in germination then it should be (a) inhibited by D-alanine and (b) be active on L-alanine analogs which act as germinating agents. In Table II

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TABLE II

RATE OF AMMONIA RELASE FROM L-AMINO ACIDS

| L-*Amino acids* | *Rate of ammonia release* $\mu$*moles/h* |
|---|---|
| Alanine | 2.46 |
| Valine | 1.42 |
| Cysteine | 1.12 |
| Serine | 0.82 |
| Threonine | 0.56 |
| Isoleucine | 0.46 |
| Leucine | 0.37 |

Clean, heat-shocked spores (25 mg) were incubated at 30° in Conway diffusion units containing 5 $\mu$moles of the indicated amino acid in 2 ml of 0.057 *M* phosphate buffer, pH 7.0. Corrections were made for endogenous (amino acid omitted) release of ammonia. Thirteen other L-amino acids were not deaminated.

some of the L-amino acids which initiate germination and $NH_3$ in these spores are shown. The deamination of L-alanine as well as the other L-amino acids is competitively inhibited by the D-isomer. A survey of some of the substrate specificities of analogs substituted in the $\beta$-carbon are shown in Table III. As can be seen the H of the $\beta$-carbon can be substituted by alkyl groups or by —OH. L-alanine is itself the most active. For comparison purposes the germination specificities of *B. subtilis* spores studied by Woese *et al.*[48] are included. Although many parallels exist, several important differences are observed. As further supporting evidence that these analogs are acting on the same enzyme, D-alanine was found to be a competitive inhibitor for all of the compounds tested, and to have identical affinity constants to that found in inhibiting L-alanine dehydrogenation. Further specificities are summarized in Table IV. As can be seen substitutions on the $\alpha$ carbon lead to loss of enzyme affinity but not necessarily of germination. More particularly $\beta$-$NH_2$ com-

TABLE III

COMPARISON OF L-ALANINE ANALOGS AS GERMINATING AGENTS AND SUBSTRATES OF L-ALANINE DEHYDROGENASE

Specificity of alanine dehydrogenase expressed as $V_M$ determined by incubating dehydrogenase at 25° with 2 $\mu$moles of DPN and L-alanine analogs, as indicated, in 0.1 *M* carbonate–bicarbonate buffer, pH 9.4. Reaction rates were measured by spectrophotometric determination of DPNH formation at 340 m$\mu$.

| *Substitutions on the β-carbon* | % *Rate of* L-*alanine Substrate* | *Germination** |
|---|---|---|
| None | 100 | 100 |
| —$CH_3$ | 32 | 65 |
| —$CH_2CH_3$ | 17 | 70 |
| —$CH_2SCH_3$ | 0 | 12 |
| $<^{CH_3}_{CH_3}$ | 2.8 | 60 |
| $<^{CH_2CH_3}_{CH_3}$ | 4 | 60 |
| —$CH<^{CH_3}_{CH_3}$ | 4.4 | 10 |
| $<^{CH_3}_{OH}$ | 0 | 0 |
| —OH | 2.5 | 0 |
| —Phenyl | 0 | 0 |

* Specificity of germination taken from studies [48] on *B. subtilis* germination.


pounds are non-substrates but germinating agents. The first of these, β-alanine, also does not complex with the enzyme or inhibit L-alanine oxidation. This is particularly important since Woese *et al.*[48] observed that germination in the presence of β-alanine is inhibited by D-alanine.

These differences may be dismissed by the fact that the observations on germination were based on findings with

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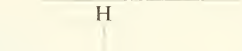
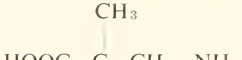
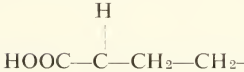
TABLE IV

COMPARISON OF L-ALANINE ANALOGS AS GERMINATING AGENTS AND SUBSTRATE OF L-ALANINE DEHYDROGENASE

(Experimental procedure was the same as that for Table III)

| *Substitution on the α-carbon* | *% Rate of L-alanine Substrate* | *Germination* |
|---|---|---|
| None | 100 | 100 |
| $-CH_3$ by $-H$ | 0 | 0 |
| $-NH_2$ by $-OH$ | 0 | 0 |
| $-H$ by $-CH_3$ | 0 | 65 |
| $\beta-NH_2$ compounds | | |
|  | 0 | 65 |
|  | 0 | 75 |
|  | 0 | 65 |
|  | 0 | 50 |

*B. subtilis* while ours came from *B. cereus*. We are inclined not to believe this, partly from the fact that the L-alanine dehydrogenase from *B. cereus* seems identical to the one described by Piérard and Wiame[46] in *B. subtilis* vegetative cells and also the evidence available with *B. cereus* indicates a similarity with the previous work on *B. subtilis*.

In considering the specificity of the initial interaction in germination, one point we must carefully keep in mind—that there are probably several alternate ways to initiate germination. Thus for example, L-alanine can be replaced by glucose for the germination of many species of spores. Also, the initial stages in germination involve a degradation of the exosporium liberating among other components L-alanine. This can be seen by blocking overall germination with arsenite and showing a release of endogenous alanine following exposure to exogenous L-alanine. Alanine liberated thus could act as an endogenous germinating agent, this germination being inhibited by D-alanine. Possibly this may be the mechanism of action of $\beta$-alanine—acting as a stimulant for L-alanine liberation.

The effect of these agents can be interpreted therefore in terms of the model shown in Fig. 8. The arguments supporting the L-alanine dehydrogenase as the initial binding site can be summarized as follows:

1. Alanine deamination is an essential but not sufficient step for germination;
2. Activation parallels activation for germination;
3. $NH_3$ release parallels germination;
4. The conditions for optimal enzyme activity (high pH) are consistent with those for optimum germination:
5. Both the enzyme and germination are inhibited by D-amino acids, especially alanine;
6. There is a parallel specificity between enzyme and germination.

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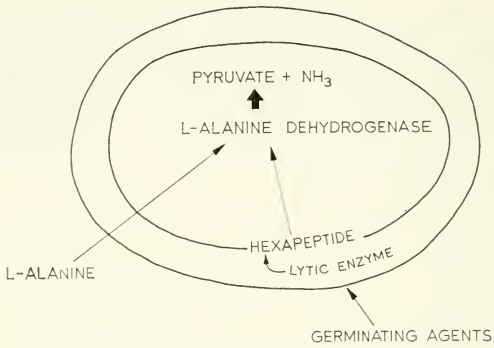


Fig. 8. Route of L-alanine for germination.

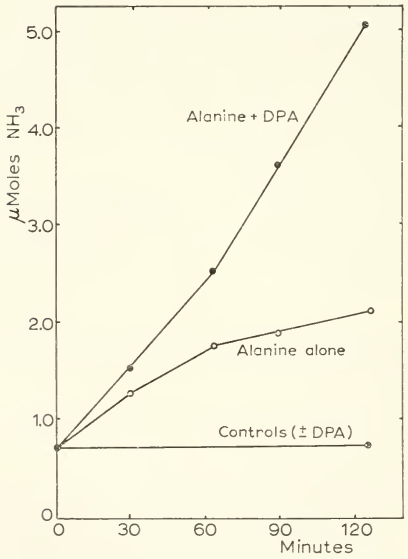


Fig. 9. Stimulatory effect of dipicolinic acid on alanine deamination by crude spore extracts. Dialyzed crude extract (2.1 mg protein) of heat-shocked spores incubated at 30° in Conway diffusion units containing 0.5 $\mu$mole of DPN, and, where indicated, 50 $\mu$moles of L-alanine and/or 40 $\mu$moles of DPA in 0.067 *M* phosphate buffer, pH 7.5.

Although the data are as yet incomplete, it is reasonably safe to conclude that the initial binding site is identical to or very similar to the L-alanine dehydrogenase. One difficulty remains in invoking its operation in L-alanine deamination—the equilibrium constant for the reaction ($K_{equiv.} = 1.3 \times 10^{-14}$) is in favor of amination[34]. In vegetative cells this enzyme is probably the route of alanine synthesis. L-alanine utilization in spores may yet be possible if the end products of the reaction, pyruvate and DPNH are rapidly utilized, thus driving the reaction to the right. As we have previously mentioned, pyruvate is metabolized by spores but the rate is low. The oxidation of DPNH by the DPA-stimulated soluble DPNH oxidase seems more likely. This was tested by measuring the rate of $NH_3$ release from L-alanine in dialyzed extracts of activated spores[34]. The results (Fig. 9) show that the rate of deamination is dramatically stimulated by DPA. DPA has no effect on the purified enzyme, and since the DPNH oxidase is present in these extracts, its role is undoubtedly that of recycling DPNH to DPN and thus keeping a low level of DPNH present in the extract.

The release of DPA during activation, therefore, can accelerate the production of pyruvate via its stimulation of the DPNH oxidase and thereby produce more rapidly the products of pyruvate oxidation required for overall germination.

## CONCLUSION

Our knowledge of the biochemical nature of the dormant state is as yet fragmentary. Some information has been presented indicating the role of the enzymes present in dormant and activated spores in converting the dormant state to the vegetative one. One may view with optimism the possibility of soon understanding the trigger mechanism involved in breaking the dormant state.

*References p. 94*

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## DISCUSSION

Keynan: Until recently it was assumed that dipicolinic acid was concerned with the regulation of metabolism in the spore stage. But Orin Halvorson reports that dipicolinic acid is not essential at the spore stage, since spores without dipicolinic acid have been obtained by him. They are, however, susceptible to heat. Are we to conclude then that there are several independent mechanisms controlling dormancy, only one of which involves dipicolinic acid.

Halvorson, Jr.: It all depends on what you call a spore. The structures with a low dipicolinic level, which are not heat resistant and have high respiratory activity are not spores in our view. We never did produce enough of these structures to be able to extract enzymes, but I think they are more activated. As long as no proper study of the kinetics of germination of these structures is possible, we cannot be sure that the results involve any contradiction of the earlier findings.

HALVORSON: There is some evidence that the heat-sensitive spore does not take up oxygen in the presence of glucose unless it is germinated with the usual germination ingredients. While we have not yet studied other enzyme activities, we can still describe it not as a heat-resistant spore but one on the way to it.

# HYPOBIOSIS IN PARASITIC WORMS

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Numerous examples of temporary cessation of vital physiological processes are known to occur at various stages in the life of parasitic worms. For the most part, empirical observations have been recorded without any attempt to explain their physiological basis. True diapause, which is characterised by a state of obligatory rest for a certain prolonged period, apparently does not occur in parasitic worms. Cases of curtailment or cessation of activity must be regarded as phenomena of adaptation which allow the egg or the larva to perform the stage transformation without disturbance, or the adult to survive unfavourable environmental conditions. This kind of cryptobiosis[1] has usually no predetermined limit and may end as soon as conditions become suitable.

## EGGS

Eggs of helminths are laid in various stages of development, depending on the species. In the case of undeveloped eggs, development may start only after they have been evacuated from the host and have come under the influence of atmospheric oxygen, given suitable conditions of temperature and other climatic factors, as for instance, ova of the most common human parasites *Ascaris* and *Trichocephalus*. If the eggs are introduced into an environment lacking the necessary climatic conditions or free oxygen (for instance, at the bottom of fermenting sewage tanks) embryonic development is retarded or stops. It will usually be resumed when proper conditions are restored. Not all developmental stages of the embryo are equally adapted to such interruptions; generally, more advanced embryos are better able to withstand them.

*Capillaria hepatica*, a nematode living in the liver-parenchyma

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of small rodents, provides an instructive example of retardation of embryonic development. The female lays eggs incessantly, but they are not eliminated from the liver and accumulate in its tissue in large macroscopically perceptible masses. As long as the eggs remain in the liver they do not develop. Their development starts only when they are liberated after the death of the host (often via the droppings of the cannibalistic fellow-rodents which devoured the infected animal!) and come in contact with atmospheric oxygen.

So called 'winter eggs' are practically unknown in helminths; winter interruption of embryonic development occurs in many species, but is caused not by any factor inherent in the egg but by the low temperature only. There is only one indication of winter eggs occurring, namely in the monogenetic trematode, *Dactylogyrus vastator*, living on the gills of the carp, but even this has been questioned recently. Nybelin[2] and some other authors supposed that this trematode lays 'winter eggs' which remain undeveloped throughout the cold season. Groeben[3] suggested that this species lays two kinds of eggs, those which develop immediately, and the so called 'Dauereier' which develop only after some time. Bauer and Nikolskaya[4] do not accept either of these hypotheses and assert that the cessation of development of the eggs of *D. vastator* depends on lowered temperature only.

In many species of parasitic worms, especially in cestodes and trematodes, the newly laid ovum contains a fully developed larva. The larva within the egg may be motionless or it may move freely or rotate. Such a larva either hatches early in natural surroundings entirely by its own efforts, or it remains within the shell in a quiescent state and hatches later in the intestinal tract of the appropriate host. The period of survival of the embryonated egg outside its host varies greatly in different species. Eggs of some species, for instance *Heterodera schachtii*, a parasite of sugar beet, may hatch not just during one season but over a number of years, even when derived from a single batch[5].

In most cases, ova predestined to be swallowed are thick-shelled and resistant to environmental factors. They may survive during long periods of quiescence. The egg of the common human parasite *Ascaris* serves as an outstanding example of such resistance; it can withstand most of the usual antiseptics and may remain alive and infective for as long as eight years in 4% formalin.

## FREE LARVAE

The hatching of larva depends on both external factors (temperature, enzymes of the host, etc.) and the internal mechanisms of the larva (pressure, specific enzymes dissolving the shell from within, etc.) activated by external factors. An interesting phenomenon occurs in the case of eggs of a plant nematode of the genus *Meloidogyne (= Heterodera)*. They may remain quiescent in the ground for a long period and are stimulated to hatch by some substances produced and excreted by growing plant roots[6].

In many parasitic worms the infective larvae live for a varying period outside their host, as in the case of the *miracidium* and *cercaria* of trematodes, of the *coracidium* of cestodes or several stages of the developing larva of nematodes. However, these stages, except the early nematode larvae, are not fully active physiologically. Miracidia, cercariae and coracidia do not feed or develop, they move about in order to reach a suitable host. This is a period during which biological activity is limited in function and time and it may be regarded as a kind of hypobiosis. Yet some of the nematode larvae, notably of the family Strongylidae of horses (so-called red worms) are remarkably vital, being able to live on the pasture twelve months or even longer, without change provided climatic conditions are suitable.

In some parasitic nematodes the infective larvae crawl onto grasses and remain on them until they are eventually picked up by grazing animals[7]. In some cases these larvae may slowly dry up intil they become fragile, surviving for long periods in this

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dehydrated state. When moistened they absorb water and regain their normal shape and movement (*Nematodirus sp.*, etc.).

Not all species of nematodes and also not all stages of the same nematode have the same capacity to withstand dehydration. Zavadovsky[8] has shown that very young Trichostrongylid larvae cannot withstand dehydration, while five-day old ensheathed infective stages remain viable when dehydrated for two months (at 22°). On the other hand, infective larvae of the notorious stomach nematode of the sheep, *Haemonchus contortus*, perish rapidly when desiccated[9].

Some larvae of nematodes parasitic in plants have remarkable capacity to withstand desiccation. Steiner and Albin[10] recorded reviviscence of desiccated specimens of *Anguina tritici* after 28 years and of *Tylenchus polyhypnus* (mature young females and larvae) after 39 years quiescence in herbarium specimens[11]. It should be noted that the observation of the revival of dehydrated *Anguina tritici* (formerly called *Anguinulina*) was made as early as in 1743 by Needham, and is one of the earliest records of a cryptobiotic phenomenon.

Free living nematodes usually show no dormancy during their life cycle. There are, however, coprophagous species which develop a so-called stage of persisting larvae ('Dauerlarve' of Fuchs[12]): when the larvae have consumed all available food they become transformed into a persisting stage which differs slightly morphologically from normal larvae. They are more slender and sluggish and withstand starvation. When food again becomes available they start feeding and develop along normal lines. In some species of the genus *Diplogaster* which belong to the above mentioned group, the persisting larvae crawl onto passing dung beetles, hide under their elytrae and are transported to a new place of abundant food. They may rest in a dormant state for long periods on this living transport.

Larvae of another nematode of this group, *Rhabditis coarctata*, build special elongated capsules glued to the exoskeleton of the dung beetles, and are carried in them until the beetle comes to a

suitable food source, where the larvae drop, start feeding, become mature and multiply normally[13].

## ENCYSTED LARVAE

The fully developed larvae of parasitic worms either enter actively or are transferred passively (free or included in eggs) to a suitable host. In the final or definitive host they develop to the adult stage. In the intermediate host practically all parasitic worms—some nematode species are excepted—encyst and become transformed into a higher resting larval stage. Such larvae of trematodes appear as *metacercaria*, of cestodes as *cysticercus* or *plerocercoid*, of acanthocephala as *acanthor*, of nematodes as *agamospirura*, etc. All these stages are in a state of dormancy which either ends when the intermediate host is swallowed by the definitive host, or the larvae die if quiescence is prolonged. The life span of the encapsuled stages of the helminths varies greatly; in some instances it may last for years. Usually the larvae encyst in a specific organ or tissue. Sometimes, however, they may establish themselves anywhere in the body. In most instances the encapsuled larvae do not change; they absorb just enough food from their host's tissues to maintain their state. In some cases, however, the encapsuled larvae grow and expand. The larva of the cestode *Echinococcus granulosus*, the hydatid, may develop in various mammals and in man to the size of a child's head and produce serious impairment of health.

The larvae of the nematode *Rhabditis maupasi** exhibit quiescence in peculiar circumstances. They live in their free-living stage in the soil, until they are swallowed by an earthworm. In the latter they partly remain active but without change in the protonephridia and partly encysted in various tissues. The latter part of the larvae apparently play the main role in propagation of the species. They remain quiescent as long as the host lives or until it autotomizes the hind

* Syn. *Rhabditis pellio* sensu Buetschli, 1873, nec Schneider, 1866 (14).

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part of its body in which they are concentrated[15]. When the earthworm dies, the larvae are liberated by disintegration of its body and use its decaying tissues as nutrients. They maturate and multiply on the spot as long as the remains of the host last and eventually their larvae disperse to start the new life-cycle in another earthworm[16].

Some encysted larvae (or their unencysted homologues living free in the tissues) can re-establish themselves if they are swallowed by an animal which is unsuitable as a final host. Thus, many insectivorous animals harbour a great number of re-established larvae which were originally encysted in insects; for instance, larvae of the dog nematode, *Spirocerca lupi*, larvae of the cat cestodes of the genus *Diplopylidum*, etc. Some carnivores may in this way acquire a great number of tetrathyridia (= larvae of the cestode genus *Mesocestoides*) which originally developed in mice which served as prey. Big predator fish may accumulate a great number of plerocercoids (= larvae of the cestode genus *Diphyllobothrium*) which originally developed in small fish. All these re-established larvae remain in the new host in their original undeveloped resting stage.

### RESTING OF NEMATODE LARVAE IN THE UNBORN FOETUS

An organism may, under certain circumstances, acquire its parasites *in utero*. Numerous examples of prenatal infection are known in various animal groups. (For example, infection of tick's brood with piroplasms, human prenatal infection with toxoplasma, etc.). Several helminths may infect their hosts in this way. Two species of nematodes, namely, *Neoascaris vitulorum* in calves and *Toxocara canis* in the dog, are the best known examples, for they show special predilection for the intrauterine route of infection[17]. The eggs of these parasites containing larvae in the infective stage must be swallowed by the host; the larvae migrate from the intestine to the venous system and reach the lungs. A few burst into the alveoli of the lung and from there, via bronchi and trachea, they reach the pharynx where they are

swallowed and reach the stomach and intestine (for the second time) to settle there. During this migration the larva moults and develops. When it has returned to the intestine after migration it is in a more advanced developmental stage than when it hatched from the egg.

The majority of the larvae, however, pass through the capillary mesh of the lungs and enter the arterial system. They are then carried to various organs and tissues where they are encapsuled, and eventually die. However, if the host happens to be pregnant, a proportion of the larvae find their way to the placenta, traverse it and concentrate in the lungs of the foetus. In this case an interesting phenomenon occurs: contrary to what happens in the adult host, the larvae do not move from the lung but remain there until the young animal is born. Presumably lack of oxygen, the necessary stimulus for the continuation of migration, prevents the larvae from moving. After birth, when the young animal begins to breathe, the larvae leave the lung and reach the stomach and intestine by the normal route. This interruption of the normal course of migration by a more or less prolonged resting sojourn in the lung of the foetus may be regarded as a kind of cryptobiosis.

## HISTOTROPIC PHASE OF NEMATODE LARVAE

It has been long observed that in the case of some intestinal nematode infestations, the larvae do not develop in the lumen of the intestine of the definitive host, but enter the pits of the gastric or intestinal glands where they are sheltered and complete their last moulting. They then usually return to the intestinal lumen and develop to adults. Kotlan[18] proposed the name 'histotropic phase' for this phenomenon.

Numerous nematodes pass through this developmental phase, *e.g.* species of the genera *Trichocephalus*, *Ascaridia*, *Trichonema*, *Hyostrongylus*, *Trichostrongylus*, etc. In his latest paper on this subject, Sommerville[19] has shown that the larvae of the sheep nematode *Ostertagia circumcincta* enter the glands in the

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abomasum, where they perform the last moult, but only a part of them emerge immediately afterwards into the intestinal tract. The majority remains in the mucosa for as long as eight weeks, and some of them eventually die in this site without emerging from the nodules which have formed around them.

The physiological significance of this phenomenon is not quite clear (there are nematode larvae which develop perfectly without it). Among several explanations proposed, it has been stressed[20] that this 'temporary burrowing' may have an evolutionary significance, suggesting a step in the direction of a more complicated migration via the circulary system as is characteristic of the genera *Ascaris* and *Ancylostoma;* alternatively, it may be regarded as a relict of such a migration.

The larvae of the horse nematode, *Strongylus vulgaris* are another example of retarded histotropic phase. During their development they accumulate in the root of the anterior mesenteric artery where they may stay for a long period and undergo only slight changes. The route by which the larvae reach this site is a matter of controversy. Their presence inside the artery is connected with the formation of aneurism and thrombosis which may cause grave consequences to the host.

### HYPOBIOSIS OF WORMS DUE TO HYPOBIOSIS OF THE HOST

As a rule, adult parasitic worms live in uniform and constant conditions inside or upon their hosts and their life goes on uninterruptedly. In some instances, however, these conditions change and the parasites mostly perish. In rare cases, the parasitic worms are able to adapt themselves to new conditions. The most striking instances of such change occur during hibernation or aestivation of some vertebrates. Only a few relevant observations have been recorded. Van Beneden[21] and Markova[22] observed changing relationships in bats, Dubinina in frogs and land tortoises[23, 24]. It appears that the reaction of the parasites to the changing physiology of the host is different in different species. The trematodes become lethargic until the hosts have returned

to the normal state. The frog nematodes of the genus *Cosmocerca*, as well as the land tortoise nematodes of the genus *Tachygonethria* slow down their physiological processes but remain active.

Simitch and Petrovitch[25] observed in Yugoslavia, a reaction of parasitic worms in hibernating spermophils *Citellus citellus*. These mammals burrow in ground holes in the autumn to spend the whole winter season in sleep; however, they often wake for short intervals. During hibernation, their temperature falls approximately to that of the surroundings, but it returns to normal rapidly on awakening. It appears that parasitic worms vary in their sensitivity to these intermittent falls in temperature and each species is able to tolerate it for a different period. Some worms always die during the hibernation of the host. The acanthocephalan *Macracanthorhynchus hirudinaceus* proved to be the most sensitive, dying after the shortest period of reduced temperature in the host. This worm is normally a parasite of the pig and is not well adapted to the physiological conditions of the spermophil. The cestode, *Hymenolepis nana* and the nematodes *Streptopharagus kutassi* and *Trichostrongylus sp.* are also not specific parasites of the spermophil, and remain alive for no more than 10 days of continuous hibernation. On the other hand, the cestode *Hymenolepis diminuta* var. *citelli* and the acanthocephalan *Moniliformis moniliformis* can withstand 20 days and the nematode *Gongylonema longispicula* even 30 days of continuous hibernation of the host. Encysted, *i.e.* resting larvae of two cestode species proved to be the least sensitive as they survived under all conditions.

Trematode larvae developing in snails may, under certain circumstances, interrupt their physiological activity while their hosts undergo aestivation, *i.e.* at a relatively high temperature. Barbosa and Coelho[26] demonstrated that the Brasilian water snail, *Australorbis glabratus*, infected with immature sporocysts of the human parasite, *Schistosoma mansoni*, is able to survive the dry season in a state of inactivity on the dried bottom mud for prolonged periods. The development of the sporocyst is then interrupted, but is resumed at the start of the rainy season when

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the snail again becomes immersed in water. (However, if the sporocyst is mature, the snail dies). Similar observations were made by Barlow[27] in Egypt on the snail *Bulinus truncatus* harbouring sporocysts of *Schistosoma haematobium*.

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# HYPOBIOTIC PHENOMENA IN FUNGI AND THEIR SIGNIFICANCE IN PLANT PATHOLOGY

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The aim of this paper is to discuss some significant aspects of hypobiotic phenomena in fungi mainly from the plant pathologist's point of view. In order to define the scope of this discussion it seems to be necessary to clarify some basic concepts. It is suggested to use the term hypobiosis for resting stages in the life cycle of the fungus which exhibit two different biological phenomena. Hypobiosis can be expressed as latent existence of the organism, imposed by adverse environmental factors and lack of proper nutrients. The organisms can be easily reactivated by applying favorable conditions answering the requirements of the fungus. In this case hypobiosis constitutes an important facet of the protective potential of the species. In contrast to this concept of latent existence, Gottlieb[1] and Garrett[2] deal primarily with obligate dormancy, involving only the innate state of the cell. A spore is dormant when it does not germinate under the same nutritive and environmental influences which later allow production of germ tubes[1]. In many instances, the present state of knowledge does not permit distinction between these two processes. For this reason it is preferred to deal with the resting phases of the fungus life cycle as one general problem.

Determining and understanding the resting period of the fungus, its duration, dependence on genetic factors and environmental conditions are of paramount importance in the control of plant disease by agronomic procedures such as crop rotation, time of planting, plowing, etc. Resting states, such as spores, sclerotia, resting hyphae, and rhizomorphs are important in enabling the pathogen to survive from one growing season to another. The organisms may remain in a hypobiotic stage when a congenial host is absent: the ideal resting state functions as

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a bridge between the appearance of susceptible crops. In some cases activation of the organisms is timed properly and secures persistence of the pathogen. In others, genetic and ecological factors may offset this timing. 'Nature's imperfection is here man's opportunity'[2]. Such an imperfection may appear in two ways: obligate dormancy may operate against the ability of the fungus to utilize suitable media that are discontinuous in their sequence, or on the other hand, response to stimuli in the absence of congenial hosts may endanger the survival of the pathogen. In this respect, the so-called 'decoy plants'[2] which stimulate the activation of the resting bodies but do not support their further perpetuation and propagation, may greatly contribute to reducing the potential inoculum in the soil. Introduction of these decoy plants into crop rotation affords an effective means for controlling root rots. Macfarlane[3] showed that some plants resistant to the clubroot disease agent *Plasmodium brassicae* may cause germination of its resting spores and thereby reduce their population in the soil. Not less interesting is the fact that oöspores of the parasite respond to stimuli of plants taxonomically remote, such as *Tropaeolum majus*, *Reseda odorata*, *Papaver rhoeas*, *Lolium perenne* and others.

## RESTING STATES IN FUNGI

### *(a) Resting spores*

Of all resting structures produced by the fungus, resting spores have been most thoroughly investigated. It seems to be a matter of general agreement[1, 4] that one of the features some spores which exhibit long dormancy have in common is their association with cytological processes, such as nuclear fusion, or fusion and division. Oospores of Phycomycetes, ascospores of various fungi, teliospores of smuts and rusts, and basidiospores in many Hymenomycetes serve as illustrations.

Thick-walled resting spores of Phycomycetes formed by sexual reproduction account for the protracted survival of the fungi in soil in the absence of a susceptible host. According to Schaffnit[5]

potato crops were attacked by the parasite *Synchytrium endobioticum* on field plots that had been kept fallow and free from weeds for more than ten years[6]. Oöspores of *Peronospora schleideni* germinate only after several years[6]. Gibbs[7] reported the survival of resting spores of *Plasmodiophora brassicae* for five years. According to Walker[8], soils are known to be infested for ten years and longer by this parasite in the absence of the host plant. For these reasons crop rotation is of limited value in controlling this disease. Stakman and Harrar[9] report that teliospores of *Puccinia graminis* formed in the late summer do not germinate until spring thus securing the winter survival of the parasite. Murphy[10] reported that some physiologic races of oat crown rust differ markedly in their precocity of telial development. Telial formation is hastened by adverse growing conditions[11]. Wahl and Tobolsky (unpublished data) proved that oat stem rust, race two, produces telia readily on coleoptiles of a number of oat varieties, while races one, six and eight are devoid of this ability. Hingorani[11] proved that resistance of teliospores of *P. graminis avenae* to activating stimuli varies with physiological races. Teliospores of some races of *Ustilago striiformis-stripe* smut of several grasses requires after-ripening periods varying from 110 days to as long as 265 days[9].

Complications in effective disease control caused by prolonged spore dormancy can be well illustrated in the case of wheat dwarf bunt incited by the fungus *Tilletia controversa*. While the ordinary bunt or stinking smut may be successfully controlled by chemical treatment of the seed, this does not hold true for dwarf bunt, since the spores of this fungus can remain in obligate dormancy in the soil for over two years[12]. It should be stressed that spores produced independently from the aforementioned cytological processes may also act as resting bodies to assure the persistence of the pathogen in an adverse environment. Park[13] found that *Botrytis*, *Trichoderma* and *Stemphylium* are capable of forming true chlamydospores under inimical conditions, and he concludes that the development of chlamydospores is associated with the ability to remain viable under

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conditions which would be lethal to fungi which lack the capacity to produce such forms. Venkat Ram[14] cited evidence that antibiotics produced by bacteria induce chlamydospore formation in *Fusarium solani*.

Several types of treatment have been employed to break dormancy. One of the methods is based on simulation of conditions prevailing in natural habitats, and consists of altering temperatures and humidities, a procedure widely used for rusts and smuts[1, 9]. It is postulated that by freezing and thawing, drying and wetting, the permeability of the spore wall becomes increased. Similar changes in permeability of the cell wall have been reported with *Ustilago striiformis* submerged in dung infusion[15]. Another method involves treatment with various chemicals—inorganic and organic acids, chloroform, ether benzaldehyde, silicylaldehyde and others[1, 9]. In this connection, Stakman and Harrar[9] pose a fundamental question and commented upon it as follows: 'How do the various classes of chemical substances produce their effect? They are an aid to experimentation, but how does nature substitute for them? In some cases at least it has been shown that the effect is not on the permeability of the spore wall. Many of the substances are known to reduce the surface tension of water, and it is possible that they may act on the content of the spore in such a way as to permit greater hydration.' The author of this paper failed so far to induce germination in teliospores of oat stem rust despite the fact that water could be introduced into the spore cells.

High temperatures have also been successfully employed in breaking dormancy of resting spores or chlamydospores[1, 16]. Goddard and Smith[17] attribute the effect of high temperature shocks to activation of carboxylase. The respiratory block is then the inactivity of this enzyme.

It should be pointed out that activation achieved by high temperature shocks can be duplicated by furfural treatment[18, 19]. These methods achieve cell activation by putting into operation different biological mechanisms, *e.g.*, heat treated spores of *Neurospora crassa* can be deactivated by exposing them to

aerobic conditions, while furfural-induced activation is irreversible[18].

Various nutrients have been recognized as indispensable factors in conditioning cell activation. Resting spores of many fungi are deficient in the ability to synthesize amino acids or vitamins and require external supply of these materials. Ryan[20] showed that the amino acids leucine, lysine and proline brought about spore germination in Neurospora mutants deficient in these materials.

The conidia of *Glomerella cingulata* have, according to Lin[21] special nutritional requirements for germination. A very low rate, or absence of germination was observed in distilled water or glucose solution not containing inorganic material, such as magnesium and phosphorus. Additional instances attesting to the importance of nutrients in germination have been cited by various authors[1, 4, 22, 23]. There are indications that spore germination may be facilitated in the case of vitamin-deficient fungi by supplying the vitamins in question[23]. The percentage germination of spores of a mutant strain of *Fusarium fructigenum* is correlated to the thiamine content of the spores[22].

Germination may be greatly accelerated by the presence of actively metabolizing plant tissues. The actively metabolizing tissue may belong to higher plants or to fungi distinctly different from, or the same as the fungus forming the spores under consideration. According to W. Brown[24], spores of *Botrytis cinerea*, *Monilia fructigena* and of several other fungi germinate more profusely in drops of distilled water placed on certain plant parts of the host and non-host, than in distilled water alone. Leach[25] demonstrated a similar effect with the spores of the bean anthracnose fungus *Colletotrichum lindemuthianum*, and Noble[26] obtained 85 to 98% spore germination of *Urocystis tritici* with the aid of uninjured seedlings of wheat or the non-host rye. The activation required presoaking of the spores for three or four days. Christensen's[27] experiments prove that spores of *Helminthosporium sativum* failed to germinate when kept in distilled water for more than one year, but germi-

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nated readily when pieces of barley tissue or sucrose were added.

In the case of *Phycomyces blakesleeanus*, Robbins *et al.*[28] contend that certain Z factors are essential for spore germination: one of these factors has been identified as hypoxanthine.

Fries[29, 30] obtained spectacular spore germination of some species of Boletus and other Hymenomycetes by sowing the spores on malt agar with cultures of *Torulopsis sanguinea*. None of the seven species of Boletus investigated germinated on malt agar in the absence of the yeast organism. Fries also found evidence that extracts of Boletus hastened spore germination of the same species.

Great difficulties have been encountered in germination of basidiospores of the common mushroom *Psalliota bispora*. Many attempts to induce germination in order to improve commercial strains by selection of monospore cultures resulted in failure until Ferguson[31] succeeded in obtaining a high rate of germination by placing spores in the proximity of the growing mycelium. De Zeeuw[32] demonstrated later that a similar stimulatory effect can be achieved if spores are transferred to agar media on which mycelium of the common mushroom fungus has been cultivated.

To sum up the discussion on the role of germination activators, R. Brown's[33] opinions may be of special interest. He advances the hypothesis that different activators operate in the stimulation of various dormant tissues, and that each activator may originate from a large number of species. At least in some cases the activators play an important part in the metabolism of the stimulated and stimulating tissue.

Attention has been called to differences between dormancy and maturation[4]. Stakman and Harrar[9] refer to the latter phenomenon as apparent dormancy as contrasted with real dormancy. They emphasize that spores may not be ripe until they are liberated naturally. This was demonstrated with the basidiospores of *Pleurotus corticatus* in which only naturally discharged spores germinated readily[9]. Similar observations have been reported with spores of *Pseudopeziza trifolii* and *P. medicagnis*[34].

Cochrane[4], Garret[2] and Gottlieb[1] do not see essential differences between dormancy and maturation. If the resting period is 'relatively short', we speak of maturation; if it is, on the contrary, a matter of weeks or months, the spore is said to have a dormant stage or resting period[4].

In some instances maturation seems to be associated with the completion of cytological processes, while the subsequent resting period involves physiological after-ripening. According to Blackwell[35], oöspores of *Phytophtora cactorum* are unable to germinate when they are first shed. Their nuclear fusion is delayed until three to four weeks after shedding, a further period of after-maturation lasting six to seven months follows the nuclear fusion. This period of after-ripening can be reduced by freezing, in contrast to the pre-fusion phase which is strongly fixed.

Spores that do not need after-ripening may fail to germinate because of the presence of certain inhibitors liberated by the spore-forming fungus. Rotem (personal communication) observed that conidia of *Alternaria solani* do not germinate while attached to the conidiophores, but germinate readily even when artificially separated from the mycelium. Self-inhibition has been reported for a number of fungi, such as *Uromyces phaseoli*[36], *Puccinia graminis tritici*, *Aspergillus niger*, conidia of powdery mildew fungi, and other spores listed by Cochrane[4]. Accroding to Forsyth[37] uredospores of *P. graminis tritici* emit trimethylethylene which inhibits spore germination. Allen[38] determined several properties of the substance presumably responsible for the inhibition of germination in uredospores of *P. graminis tritici*.

*(b) Cryptobiosis in multicellular resting bodies*

Unlike spores consisting of one or a few cells, the resting bodies discussed in the following are multicellular. Their biology and survival ability are of paramount interest to the plant pathologist. Some of the pathogenic fungi capable of forming sclerotia, rhizomorphs or resting hyphae make disease control

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extremely difficult[39]. According to Stover[40]: 'The majority of root-infecting fungi do not have an obligate type of dormancy as defined by Gottlieb. The 'dormancy' of most root-infecting fungi is enforced by adverse environmental conditions.' Table I* redrawn from Stover's paper demonstrates the relationship between the survival ability of the reproductive resting structures and plant rotation schedules employed for controlling some of the economically important diseases produced by root-infecting fungi. The role of microsclerotia in the persistence of the *Verticillium wilt fungus* was demonstrated by Wilhelm[41]. He proved that the sclerotia of this pathogenic organism retain viability for 13–14 years.

Two sclerotia-producing pathogens. *Sclerotia rolfsii* and *Sclerotinia sclerotiorum* cause serious losses to agricultural crops in Israel.

Reduction in yields inflicted by *S. rolfsii* becomes very pronounced in summer. Stakman and Harrar[9] stress the great diversity in vitamin requirements for sclerotia production among a relatively small number of single basidiospore isolates. The vitamins involved are: thiamin, biotin and nicotinic acid.

Bedi[42, 43] studied sclerotial production of *S. sclerotiorum* extensively. He demonstrated genetic variability among strains of this organism as far as sclerotial formation is concerned. Sclerotial formation may be stimulated by staling products of the same organism by low temperature and by chemicals such as uranium nitrate[9]. Sclerotial formation by a non-sclerotial mutant can be induced by growing it along with a sclerotia-producing isolate. Fully mature sclerotia floated in water begin to germinate after 32 days while germination commences after 16 days in sclerotia previously subjected to ultra-violet radiation.

* Table I is reprinted by permission of the copyright owners, the Regents of the University of Wisconsin, from H. R. Stover, Plant Pathology: Problems and Progress, 1908–1958, the University of Wisconsin Press, Madison, Wisc., U.S.A., 1959, p. 340.

TABLE I

REPRODUCTIVE STRUCTURES, LENGTH OF SURVIVAL IN SOIL, AND LENGTH OF ROTATIONS REQUIRED TO CONTROL GENERA OF ROOT-INFECTING FUNGI OF MAJOR ECONOMIC IMPORTANCE

| *Major pathogen genera* | *Type of reproductive structures produced in nature* | *Main reproductive structures surviving in nature* | *Years of known survival in soil in absence of preferred hosts* | *Years of rotation required for control* |
|---|---|---|---|---|
| Armillaria | basidiospores, rhizomorphs | rhizomorphs | 2–5 | 3–4 |
| Fomes | basidiospores, rhizomorphs | rhizomorphs | 2–3 | 3–4 |
| Fusarium | micro and macroconidia, chlamydospores | chlamydospores | 5–15+* | 4–6 |
| Helminthosporium | conidia | conidia | 3 | 2–3 |
| Ophiobolus | conidia, ascospores | ascospores | 2–4 | 2–3 |
| Phymatotrichum | sclerotia | sclerotia | 2 | 2–3 |
| Phytophthora | sporangia, zoospores, oospores, chlamydospores | oospores | 2–8 | 2–5 |
| Plasmodiophora | zoospores (amoeba), chlamydospores | chlamydospores | 3+ | 4–6 |
| Pythium and Aphanomyces | sporangia, oospores, zoospores | oospores | 5+* | 2–3 |
| Rhizoctonia (Corticium) (Pellicularia) | sclerotia, conidia | sclerotia | 5+* | 2–3 |
| Sclerotinia | sclerotia, ascospores | sclerotia | 5+* | 2–3 |
| Sclerotium (Macrophomina) | sclerotia, conidia | sclerotia | 5+* | 2–3 |
| Spongospora | zoospores (amoeba), chlamydospores | chlamydospores | 3–5 | 3–5 |
| Synchytrium | zoospores, sporangia (chlamydospores) | chlamydospores | 6–8 | 8 |
| Thielaviopsis | conidia, chlamydospores | chlamydospores | 5+* | 3–4 |
| Tilletia | basidiospores, chlamydospores | chlamydospores | 1–2 | 1–2 |
| Verticillium | sclerotia, conidia | sclerotia | 5–15* | 5–6 |

* Some species of these genera are soil and litter inhabitants that appear to survive for indefinite periods in some soils, and rotations frequently yield variable results.

Unpublished data obtained in our laboratory show that production of sclerotia by laboratory cultures in *Rhizoctonia solani* could be conspicuously increased by inoculating the nutrient medium with *Bacillus subtilis*. It was further ascertained by our studies that *Mycosphaerella pinodes* and *Helminthosporium teres* which incite respectively the most severe diseases of peas and barley in Israel, pass through the summer in this country as a resting mycelium. Most probably many powdery mildew parasites which affect various crops in Israel, as well as *Plasmopara viticola*, the cause of downy mildew on grapes and other important plant pathogens survive adverse conditions here as resting mycelia.

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## DISCUSSION

Keynan: You mentioned that spore germination occurred on a special variety of growing yeast; can this be replaced by yeast extract?

Wahl: It may be possible, we did not try it, however, other yeast varieties did not give the same effect.

Mayer: I have heard of a case of uredospores being stimulated by coumarin, are any other cases known? What is known of the properties of sclerotia, and what characterizes them as dormant organs?

Wahl: Van Sumere[1] and his associates ascertained that coumarin, *o*-coumaric acid, indolacetic acid and some other compounds cause a marked stimulation of the uredospores'

germination; the mode of action of these substances is not clear. Your second question is rather difficult to answer, since different fungi are capable of producing sclerotia as a result of diverse stimuli. It is assumed that in many instances sclerotia formation is induced by inimical environmental factors. Sclerotia resist adverse conditions, for example Avizohar-Hershenzon (personal communication) demonstrated that sclerotia of *Sclerotium rolfsii* are tolerant of relatively high temperatures prevailing in alimentary tract of cows, but lose to a considerable degree their viability in compost heaps. Lavee[2] reported that coumarin, maleic hydrazide, and triethanolamine bring about pronounced inhibition in sclerotia germination of *Sclerotium rolfsii* and reduce the number of sclerotia per culture.

KEYNAN: It is well known that the soil contains a large number of spores of fungi which will only germinate under favourable conditions. Inhibitors have been shown to exist in the soil, is anything known about these?

WAHL: I presume that you have in mind the phenomenon referred to as fungistasis. Investigations in various countries proved that spores of many fungi rest dormant in soil but germinate readily when placed in distilled water.

Dobbs and Hinson[3] postulated the widespread existence of fungistasis in soil. The nature of the germination inhibitors is obscure. The inhibitory effect can be removed by specific organic soil amendments, by soil heating, or prolonged soil desiccation. Fungistatic substances can be neutralized by root exudates of certain plants. Buxton[4, 5] found that exudates from the roots of pea varieties stimulate germination of spores belonging to *Fusarium oxysporum f. pisi* to which the varieties are vulnerable and depress that of races to which they are resistant.

Chinn and Ledingham[6] suggested that successful control of some root diseases attained by green manures application is

due to enhanced spore germination of the pathogen in the absence of the congenial host. Consequently, the produced hyphae perish.

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# INSECT DIAPAUSE IN RELATION TO THE ENVIRONMENT

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The phenomenon of dormancy is a familiar one to entomologists. Indeed, so striking are the instances afforded by insects and mites that Henneguy, as long ago as 1904, appropriated the special term diapause ('interruption of work') to describe the condition. His intention was to emphasise one of the most noteworthy features, namely the failure of growth (embryonic, larval, pupal or reproductive according to species) even under circumstances which would be expected to favour it. Although the employment of the term has served to draw attention to ecological and physiological problems associated with dormancy, its use has not been an unmixed blessing since it has led some investigators to conclude that 'diapause' is a single phenomenon and is therefore explicable in terms of one comprehensive hypothesis. Yet the examination of almost any group of closely related species reveals that their dormant stages occur at different times in the life cycle—a sure indication of their independent evolution. And recent studies on intraspecific variations have further emphasised the extraordinary plasticity of the diapause-controlling mechanism. With such a background we should be prepared for differences rather than similarities. 'Diapause' must indeed be recognised as a portmanteau term covering many types of physiological arrest.

Keilin's[1] classification of 'hypobiotic' forms of life is based on metabolic considerations. The distinction is made between 'ametabolic' organisms which remain viable without detectable respiration and 'hypometabolic' organisms in which respiration remains appreciable, although below the level characteristic of active growth. In arthropods nearly all our examples of quiescence and diapause fall under the latter heading. One of the rare

exceptions is provided by the larva of the Chironomid midge *Polypedilum vanderplanki* which colonizes temporary rock pools in northern Nigeria. In the absence of water the larvae rapidly become completely dehydrated. Yet, a few minutes after replacing these wizened objects in water, imbibition begins, pharynx and heart contractions appear and the larva begins to swim and feed. Hinton[2, 3] has shown that larvae can be desiccated and rehydrated repeatedly. When thoroughly dry (containing less than 1% water) they can be heated to 100° for short periods, to 65° for one day and will withstand several hours exposure to absolute ethanol or 77 h in liquid air. There is no measurable respiration. Although capable of resisting dry storage for over five years, they are not immortal; and after 10 years over calcium chloride larvae often rehydrate satisfactorily but fail to feed and metamorphose.

In this example dormancy is directly controlled by the availability of water. In other dormant or diapause states the relationship between the environment and the onset or termination of the dormant state is more indirect. The relevant component, whether photoperiod, temperature or nutrition, acts on the tissues through an intermediate physiological mechanism, namely the endocrine system. I shall consider the question of receptor structures, the role of hormones in the control of diapause and biochemical events in the tissues in my second lecture. At the moment I wish to concentrate on the types of environmental 'cues' which are utilized by arthropods for controlling their dormant periods and comment briefly on the type of physiological information which can be secured by studying these external agencies.

## INDUCTION OF DIAPAUSE

A considerable array of factors, differing according to species, are concerned in controlling the onset or induction of diapause. In temperate climates *photoperiod* is one of the most important. Many insects and mites can be said (by analogy with plants) to

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have a long day response, since diapause is prevented in long days and induced in short days. The moth *Acronycta* and the red spider mite *Panonychus ulmi* belong to this group[4, 5]. The response curve shows a characteristic high rate of change at the critical photoperiod, which ranges from about 13 h of light daily in insects from southerly latitudes to 20 h in insects from the extreme North. In at least a few insects, however, this general pattern is reversed. In such 'short day' species a short photoperiod prevents diapause. The commercial silkworm *Bombyx mori* is the best known example. Others include the Desert Locust *Schistocerca*[6] and the leafhopper *Stenocranus*[7]. It is noteworthy that the diapausing and non-diapausing forms of the latter species are strikingly heteromorphic.

Since these and many other light-responsive species are phytophagous, the question arises as to whether the plant serves as the insect's photoperiodic receptor. By transferring them serially between different host plants, the insects or mites can be exposed to one type of photoperiod, their hosts to another. Experiments of this type have shown that in nearly all cases the insects respond in their own right. However, the soil-dwelling larvae of the cabbage root fly *Erioistichia* appear to be an exception, for although their diapause behaviour is unaffected by placing an opaque cover on the soil round the root of the plant, the light treatment of the latter is quite influential[8]. But it is not yet known whether photoperiodic or photosynthetic events in the plant provide the 'cue'.

Some insight into the mode of action of the cycle of illumination has been obtained by varying the lengths of the light and dark periods independently. In the mite *Panonychus*, for example, there is no doubt that both light and darkness plays an integral part in the photoperiodic response, the length of the dark period being particularly critical[9]. So far as the dual significance of the light and dark phases is concerned, other arthropods seem to resemble *Panonychus:* but the details of the response differ considerably. A feature which seems to be typical of the group (and untypical of plants) is that long

inductive dark periods are not greatly affected by short light breaks. The impression gained is of a photochemical reaction—either a synthesis or more probably a breakdown process—which is opposed by a dark reaction. Both the light and dark processes seem to develop slowly. In permanent darkness an intermediate response is often obtained, some individuals entering diapause, others developing without interruption. It seems possible that in these and other unnatural conditions the endocrine mechanism which controls development is no longer fully 'geared' to the photochemical receptor process and therefore tends to 'drift' erratically.

The threshold of sensitivity to light is in the region of 0.01 ft.-candles in many insects and is less than 0.0025 ft.-candles in the midge *Metriocnemus* investigated by Paris and Jenner[10]. An important part of the twilight in temperate latitudes therefore comes within the range of the photoreceptors.

*Temperature* is nearly always an important factor in the control of diapause. As a rule high temperatures augment long photoperiods and low temperatures short ones. This means that in long day insects high temperatures tend to prevent diapause, while in short day insects, such as *Bombyx*, these conditions favour diapause.

The modifying role of temperature gives some indication of the possible origins of obligatory and facultative diapause[11]. Many insect varieties or sub-species differ in their diapause characteristics. In *Bombyx*, for example, there are one-generation (univoltine) races with an obligatory diapause, 2-generation (bivoltine) races with a facultative diapause; and multivoltine races which are virtually diapause-free under any environmental conditions. These races may have arisen through selection of the temperature responses. Obviously, if the temperature at which the long- or short-day photoperiodic stimulus can induce a differential response is shifted beyond the normal developmental limits, diapause would either be prevented entirely or would occur under all environmental conditions. A logical inference is that in insects with obligatory diapause the mechanism for perceiving these factors may still be functional.

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*Nutrition* is of occasional significance. In *Panonychus* for example, a diet of senescent leaves or foliage damaged by the feeding punctures of other mites, exerts a strong diapause-promoting influence and can even overcome the opposite effect of a long day and a high temperature. The larvae of the codling moth *Carpocapsa pomonella* are influenced photoperiodically by light which penetrates through the flesh of the apple; but the maturity of the fruit also has some effect[12, 13].

Although, in general, *population density* has surprisingly little effect, crowding does sometimes induce diapause. This is so, for example, in the grain moth *Plodia*[14].

These factors frequently act on the insect long before growth is actually arrested. In the case of species with a pupal diapause, the sensitive period may occur during larval development, the precise instar or instars depending on the species. Egg dormancies are controlled by the maternal physiology. However, the direction of the maternal response is often decided far back in ontogeny. In *Bombyx*, for example, photoperiod acts mainly on the late embryo, while it is still enclosed in the shell. It may well be that the 'directions' provided by the photoperiod are registered in the central nervous system and associated neurosecretory centres, since these are already differentiated in the embryo. Perhaps even more remarkable are certain hymenopteran parasites *(e.g. Mormoniella)* in which the diapause stage is the fully grown larva. The temperature conditions to which the mother is exposed again determine whether dormancy will occur. But in this instance the effect must be transmitted through the cytoplasm of the egg and cannot be dependent on the continuity of any organ system[15].

## THE TERMINATION OF DIAPAUSE

In many insects and mites the state of suspended animation is so intense that the diapausing stages finally die unless the appropriate releasing stimuli are forthcoming from the environment. One gains the impression that almost any agency of

significance in the natural environment can be utilized by natural selection as an appropriate signal. Thus the hypopus of the mite *Histiostoma* (this is the extra non-feeding nymphal instar which appears when poor nutritive conditions supervene) can be induced to moult and transform into a feeding nymph by the smell of yeast[16].

However, *temperature* is undoubtedly of very general significance. The silkworm egg—in this respect a classical object of study—has a temperature optimum for the completion of diapause of about 8°, which is well below the threshold for normal development. The temperatures required by different species show a general correlation with their environments, those from temperate or cold climates usually needing a temperature in the range 0–10°, while 10–15° is often more suitable for species from warmer regions. Higher temperatures are also effective in insects from temperate climates which have an aestivating rather than a winter dormancy (*e.g.* the winter moth *Operophtera*[17]).

The precise temperature requirements for terminating diapause are complex and variable. No two species are exactly alike in this respect. The silkworm egg needs 40 or so days of chilling. But longer or shorter periods may be required by other species with a diapause of greater or lesser intensity. It is also very common to find that the temperature optimum varies during the course of diapause completion. I shall consider some of the physiological implications of this phenomenon in my second lecture.

## DORMANCY AND THE LIFE CYCLE

Although it is not always possible to estimate the precise significance of the more complex temperature requirements, it is safe to infer that they represent a successful adjustment to the average temperature conditions of the local environment. A dormant period of optimal length ensures that the active growth of the population is delayed until weather and food supplies

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are most favourable. Nevertheless, the bearing of diapause induction on the phenology can perhaps be more easily appreciated. The function here is to forestall the onset of adverse conditions. The following are some examples involving photoperiodic control.

The life cycle of the red spider mite *Panonyclus ulmi* is typical of species with several annual generations. The long daylengths of summer are responsible for the appearance of several successive generations of 'summer' females laying non-diapause eggs on the leaves. Under English conditions females laying winter (diapause) eggs appear in the penultimate and last (4th and 5th) generations in response to the reduced daylengths of September. These individuals normally deposit their eggs on the bark of the tree long before leaf fall. However, heavy damage to the foliage by large mite populations often causes premature leaf abcission. It is interesting to find that under such conditions of semi-starvation diapause is also induced prematurely, even if long day conditions are still prevailing. In this respect the response to nutrition appears as a kind of 'double assurance'.

Since day length provides a fixed 'point of reference', it might be expected that the composition of the terminal generations, and even the total number of generations in a season, would depend on the environmental temperature—a much more variable component. This has indeed been demonstrated in the moth *Polychrosis* by Komarova[18]. Although this species is always bivoltine in the southern parts of the USSR, the incidence of diapause in the second generation varies from year to year and from place to place. In a cool year or at high altitudes the second generation is delayed relative to the critical photoperiod with the result that a much higher proportion of individuals enter diapause.

I have already mentioned that some short day insects have been described. The function of this response may be simply to invert the season of dormancy. In the leafhopper *Stenocranus* for example, a long photoperiod induces a summer (aestivating)

diapause. *Bombyx*, however, has a normal hibernation and the reversed response stems from a different cause. This is connected with the early occurrence of the sensitive egg stage in spring. A reversed response is therefore necessary to prevent diapause from supervening after one generation. In species such as *Panonychus* this difficulty does not arise since the sensitive stage occurs much later in the life cycle; and in addition. dormancy is maintained until the short day spring period has been almost completed.

More complicated relationships are also known. According to Masaki[19] the Japanese magpie moth *Abraxas miranda* exhibits two separate types of pupal diapause which differ in intensity. The more intense summer diapause is induced by a moderately long photoperiod of 14–16 h, while the weaker diapause is induced in early winter by a somewhat shorter photoperiod of 11–13 h. Like other 'short day' insects, a day length of 7–9 h induces development without diapause. This response has the effect of imposing a bivoltine pattern on the annual cycle. Larvae pupating early in winter have a short dormancy whilst those doing so later on (they feed on evergreen *Euonymus*) develop without interruption. The apparent function of this adaptation is to synchronize the emergence of the moths in spring.

## GEOGRAPHICAL VARIATIONS

Since the control of diapause involves rather precise relationships with photoperiod and temperature, it is not surprising to find that the character of the diapause in widely distributed species is by no means uniform, and that the differences are determined genetically.

Diapause races are sometimes surprisingly local in their distribution. For example, there are at least three strains or races of *Locusta migratoria* in southern France alone[20]. However, other species show continuous transitions in the environmental response. Thus Danilyevsky[21] has found that the

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critical photoperiod is smoothly graded in populations of the moth *Acronycta rumicis* and ranges from nearly 20 h of light daily in the Leningrad populations to about $14\frac{1}{2}$ h in populations from the Black Sea coast. In the South moderate or high temperatures are also more effective in preventing diapause-induction by short days. The tendency in nature is therefore for the period of active growth to extend into the winter season and even to continue through it. Very similar relationships have been demonstrated in the red mite *Tetranychus urticae*[22] and in *Anopheles maculipennis*[23].

The genetic examination of this interesting material is in its early stages. But it has already been shown in *Acronycta* that $F_1$ hybrids of local photoperiodic races respond to intermediate critical photoperiods. And no clear-cut segregation emerges when the $F_1$ progeny is backcrossed with the original forms[24]. The type of inheritance is therefore polygenic.

These results immediately indicate the high adaptive value which must attach to the correct adjustment of the diapause-inducing and diapause-terminating mechanisms. They also suggest that there must be considerable selective advantage in retaining the plasticity and variability of the diapause character.

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## DISCUSSION

Galun: Since insects do not see red light, it would be interesting to compare the spectrum for photoperiod sensitivity with the light spectrum.

Lees: There is very little information on this subject although Geispits has examined various species of *Lepidoptera* from this point of view. He found that roughly the same wavelengths produced visual and photoperiodic responses and he therefore concluded that the simple eyes were in fact the photoperiodic receptors. But even if the action spectrum is identical this conclusion may not be warranted.

Galun: It could indicate that the same pigment is involved, if not the same organ.

Lees: Yes. Photolabile pterins have recently been demonstrated in the insect compound eye; and it is possible that we should think of these substances in searching for the photoperiodic pigment.

Kohn: You said that diapause is an inherited characteristic rather than a condition, but you mentioned too that environmental influences on the adult insect resulted in changes in the diapause, the larvae and the eggs. You also said that there must be a way of transferring the information from the adult to the stage in which diapause occurs. Would it be possible that the information is recorded on genetic material?

LEES: I do not think that genetic material is involved. In the silkworm *Bombyx* the late embryos are light-sensitive and the effect is finally seen in the adult moth. Thus although a long time interval is involved, it is still the same individual and no inherited effect need be invoked. In *Mormoniella* the photoperiodic experience of the mother is transmitted to her fully grown larval offspring. But in both cases the effect is confined to one generation. In the next generation the switching mechanism can again be operated in either direction by the appropriate environmental conditions.

NACHMONY: Is photoperiod only effective in inducing diapause or also in breaking it?

LEES: In some cases it is effective in both. One example is the leafhopper *Stenocranus* which aestivates in summer under long day conditions but emerges from diapause in autumn in response to shorter daylengths. A second generation is then produced. The moth *Dendrolimus* becomes dormant as a larva in autumn and then feeds and grows very little until the daylength has lengthened beyond the critical photoperiod in spring. Nevertheless, such examples are scarce. The diapause-inducing and diapause-terminating factors are usually quite different. In most species it is presumably an adaptive advantage to have separate timing mechanisms governing these two events, since it is unlikely that the optimal dates for diapause induction and termination will have the same daylength.

HESTRIN: Could you tell us more about the fly *Polypedilum* you mentioned? How was its remarkable resistance to desiccation discovered?

LEES: It was first collected by F. L. Vanderplank from shallow rock pools near Kaduna in Nigeria. During the rainy season these pools are full of water and the larvae can be readily observed. During the dry months the pools contain nothing but a small cake of dried mud. Most entomologists, I think, would pause to wonder what had happened to the insects. This problem was solved when some mud was soaked in

water. All the experimental work on this species has been carried out by H. E. Hinton at Bristol.

HESTRIN: Was any attempt made to grow this fly in the laboratory?

LEES: Hinton has attempted it, but it has proved difficult.

GALUN: Long day and low temperature induce diapause, while in short days the high temperature induces it. What happens if you artificially reverse one of the conditions, so that you induce with one element and defer with the other.

LEES: It works additively. If a long day insect is given a short day with high temperature, the latter will prevent at least some of the individuals from entering diapause. This lack of temperature compensation may be adaptively useful, since rapidly breeding species can complete an additional generation under short day conditions.

# THE ENDOCRINOLOGY AND BIOCHEMISTRY OF INSECT DIAPAUSE

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The blood-sucking reduviid bug *Rhodnius* invariably undergoes ecdysis after taking a full blood meal. In 1934 Wigglesworth[1] observed that insects decapitated immediately after the meal failed to moult, although they often remained alive for over a year. He concluded that they had been deprived of a growth hormone produced in the head. His comparison of this experimentally induced state with other naturally occurring examples of dormancy has since been shown to have a general validity.

As knowledge of the humoral control of moulting and metamorphosis increased, it became apparent that the source of the moult-inducing hormone was the brain or, more precisely, the groups of neurosecretory cells located near the mid-line in the dorsum of the forebrain. The axons of these cells were shown to end in two small organs situated just behind the brain, the *corpora cardiaca*. Droplets of secretion, presumably transporting the hormone, pass down the axons to the *cardiaca* and are there discharged into the blood.

This relatively simple picture was considerably complicated when it was shown that a further endocrine organ—the prothoracic glands—also secreted a moult-inducing hormone.

The apparent contradiction was resolved by Williams[2] who showed that the neurohormone from the brain activates the prothoracic glands. It is the hormone secreted by the latter organs—ecdyson— which stimulates the tissues to undergo a moulting cycle. Since larval and pupal diapause involves the suspension of ecdysis and metamorphosis it is not surprising that the same dual endocrine mechanism controls diapause. Indeed, the material used by Williams for demonstrating the endocrinological relationships of the brain and prothoracic

glands was a diapausing insect, the giant silkmoth *Hyalophora cecropia*. The combination of large size and tolerance of drastic surgical procedures makes this the material of choice.

When Williams removed the brains from diapausing cecropia pupae he found that they failed to develop even if chilled for the length of time which would normally bring dormancy to an end. On the other hand, when brains from chilled pupae were transplanted into unchilled pupae, development was promptly initiated. The interaction of brain and prothoracic glands was tested by implanting these organs, either separately or together, into the isolated pupal abdomen, which is without any known endocrine source of its own. Moulting and metamorphosis was only caused when both organs were present.

These results suggested that the immediate cause of diapause in the cecropia pupa was the inactivity of the neurosecretory brain cells, and that the failure of growth was due to the absence of a promotive hormone (ecdyson) rather than to the presence of an inhibitory factor. This hypothesis was tested by grafting together a chilled and an unchilled pupa. Both members of the pair developed, indicating that no inhibitory substances of any significance were present in the unchilled insect. This conclusion appears to hold good in many species. Indeed, growth inhibitors of the type found in plant seeds are unknown in dormant insects.

As might be expected, the brain-prothoracic gland system has also proved to be the main controlling system in *larval* diapause. Nevertheless, it may well be that a further endocrine—the *corpus allatum*—is sometimes involved. It is well known that in larval insects this organ produces a secretion—the juvenile hormone—which leads to the retention of the larval characteristics. Unmistakable signs of activity have been found in the *corpora allata* of some diapausing insects, for example in the larvae of the rice stem borer *Chilo*[3]. However, it is still uncertain whether these organs play any positive role in stabilizing diapause.

The humoral control of *embryonic* diapause, which has been

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studied mainly in the silkworm *Bombyx*, involves a different combination of endocrine organs, a particularly important centre being the suboesophageal ganglion with its associated neurosecretory cells. Pupae and moths, determined by their previous photoperiodic treatment to be non-diapause egg-producers, can readily be induced to lay diapause eggs by implanting ganglia from diapause egg-producers[4]. The release of the 'diapause hormone' from the suboesophageal ganglion is considerably modified by the brain, which in this instance apparently acts through the nervous paths in the circum-oesophageal commissures. It can either promote or inhibit the synthesis and release of the hormone in accordance with the previous photoperiodic treatment[5]. The *corpus allatum* also seems to play some part in diapause regulation, its effect being antagonistic to that of the suboesophageal ganglion. But here again a general control of its activity seems to be exercised by the brain[6].

Suboesophageal ganglia that are active in causing *Bombyx* to lay diapause eggs have also been obtained from certain species with a pupal diapause. Since the 'diapause hormone' is not known to be concerned in controlling pupal diapause, it seems likely that this secretion is utilized for other physiological functions. In this context it is worth recalling that the cockroach suboesophageal ganglion secretes the neurohormone responsible for initiating and maintaining the diurnal rhythm of activity[7]. It remains to be decided whether these substances have anything in common.

Although the maternal endocrine systems which control the production of the two egg types in *Bombyx* have been the subject of detailed examination, the actual mechanism which leads to the growth failure in the embryo is still obscure. There is no reason to suppose that the 'diapause hormone' from the suboesophageal ganglion is passed into the egg and acts as an inhibitor. When diapausing embryos of *Bombyx* are dissected out of the egg and are suspended in a hanging drop side by side with a non-diapause embryo, the growth of the former is

stimulated[8]. This seems to indicate that some substance required for embryonic growth is lacking in the dormant embryo.

In most locusts and grasshoppers embryonic growth cannot be completed until water is taken up from the environment. Slifer[9] has shown that diapause can be terminated in the egg of *Melanoplus differentialis* if a waxy layer is removed from the surface of the hydropyle—the special water-absorbing area of the egg-shell. The effective solvents include medicinal paraffin which is so innocuous to living tissues that any stimulatory effect through 'wounding' is extremely improbable. Yet the causal mechanisms which control the ordered appearance and disappearance of such physical and physiological barriers are still unknown. The mode of action of low temperature in bringing about the final dissolution of the wax layer is one such problem. Unlike the egg of *Bombyx*, which does not take up water, control does not seem to reside in the embryo itself, for Bucklin[10] has found that diapausing embryos, when 'explanted' in a hanging drop of Ringer, immediately resume their development.

Although the *corpus allatum* ceases to secrete juvenile hormone just prior to metamorphosis, it often displays renewed activity during adult life. Indeed, its secretion at this time has been shown to be necessary for the maturation of the eggs of many insects. Since adult diapause is characterised by reproductive inactivity, it was suspected that the *corpus allatum* might be involved. This view has been proved correct by De Wilde[11] who showed that Colorado beetles *(Leptinotarsa)* that are about to enter diapause in response to a short photoperiod can be induced to leave the soil, reverse their geotaxis, feed and lay eggs, by implanting active *corpora allata*. The *corpora allata* of this insect may also be influenced by the brain.

## MODE OF ACTION OF ENVIRONMENTAL FACTORS

In my first lecture I gave some account of the way in which environment controlled diapause induction and diapause termi-

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nation, but hardly touched on the physiological mechanisms which must be involved.

We have seen that diapause is frequently evoked by the appropriate photoperiod, temperature or nutrition. It has been shown that blinding fails to eliminate the photoresponse. Neither the stemmata (simple larval eyes) nor the compound eyes are therefore concerned. The photoreceptors have not yet been identified in a diapausing insect. Nevertheless, it is certain that in aphids with a similar photoperiodic reaction (which, however, governs a different process—the formation of virginoparous and oviparous offspring) the receptor lies *inside* the animal. When different areas on the head, thorax and abdomen were illuminated with small spots of light for the necessary time, leaving the rest of the body dark, a positive response could only be elicited from the dorsum of the brain (Lees, unpublished results). Whether the photosensitivity of this region is due to the presence of neurosecretory cells or two other light-sensitive structures (perhaps neurones), remains to be decided.

Little is known about the role of temperature; but a clue to the possible mode of action of nutrition on diapause induction is provided by the work of Johansson[12] who showed that the failure of *Oncopeltus* to mature eggs when starving is due to the inhibitory action of the brain on the *corpus allatum;* this influence disappears when the insect is fed or the allatal nerve cut. A comparable relationship in which nutrition exerts a controlling influence on the activity of the brain may exist in diapausing insects.

This information, scanty though it is, suggests that the brain may be the centre on which diapause-inducing stimuli act. Certainly, the role of the brain in terminating diapause has been proved conclusively. You will recall that unchilled cecropia pupae end their diapause when supplied with a brain from a chilled pupa. Davis and Schneiderman (unpublished results) have demonstrated that this is indeed the site of action of low temperature since brains chilled *in vitro* at 6° in a medium of haemolymph for 8–14 weeks become competent to end diapause.

Van der Kloot[13] has shown that the humoral inactivity of the brain during diapause is accompanied by a fall in the level of cholinergic substances. The progress of chilling is marked by a gradual rise in the titre of acetyl choline and finally by the reappearance of cholinesterase and the resumption of electrical activity. The virtual depolarisation of the brain neurones during diapause appears to serve the purpose of 'protecting' the neurosecretory cells either from possible sensory stimulation or from random discharges from other neurones.

Although the diapause-completing processes which take place in response to chilling may be associated with changes in the ordinary neural tissues of the brain, it may well be that the primary events take place within the specialised neurosecretory cells. If so, these processes must be extremely complex, as the following well analysed instance illustrates. Schneiderman and Horwitz[14] have shown that diapause in the hymenopterous parasite *Mormoniella* can be terminated by chilling the larvae at 5° for an adequate period. But if the chilling is interrupted by periods of warming, the effect of low temperature is largely undone. The chilling process is therefore reversible. A further complication is that the temperature characteristics of the diapause-completing process undoubtedly change with time. The first stage requires moderate warmth, although diapause can never be ended in these conditions. The second stage requires low and the final stage moderately high temperatures. Different phases in diapause completion can also be separated by studying the effects of oxygen lack. It turns out that the chilling process is aerobic, whilst the final high temperature phase is favoured by anoxia.

The action of low temperature can perhaps be most simply seen in terms of competing reactions with different temperature coefficients. The model proposed by Schneiderman and Horwitz consists of a synthetic reaction opposed by an oxidative breakdown process. Low temperature slows down the latter reaction but not the synthesis, thus permitting the brain to accumulate the substance necessary for the production of the neurohormone.

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Recent observations by Way[15, 16] on the fly *Leptohylemyia* are particularly interesting in this connection. The eggs of this species exhibit two optima for diapause termination, at approximating $+2^{\circ}$ and $-20^{\circ}$. As the latter temperature is probably incompatible with biochemical activity, Way has suggested that the physical rupture of a lipoprotein membrane—perhaps even some element of the neurosecretory cells—may be involved.

Histological methods have as yet contributed rather little towards unravelling these events. In some species, however, promising results have been achieved. The hawk moth *Mimas* is a case in point. There it has been possible to correlate the chilling process with the synthesis of neurosecretory material and to trace its passage down the axons to the *corpus cardiacum*[17].

## RESPIRATION AND METABOLISM

Diapausing insects are characterized by a low level of metabolism. In the cecropia silkworm, for example, a precipitous fall in respiration takes place as the larva pupates. Throughout the months of diapause oxygen consumption continues at a very low level, only to increase dramatically as the prothoracic gland hormone is secreted[18].

Bodine and his co-workers many years ago drew attention to the possibility that diapause is associated with some change in the respiratory enzymes, particularly the cytochrome system. This was indicated by the insensitivity of their material—the egg of *Melanoplus*—to cyanide. This view has since been confirmed and greatly extended by Williams and his associates who showed that the low diapause metabolism of the cecropia moth is also unaffected by cyanide and carbon monoxide. Since these inhibitors combine with cytochrome *c* oxidase, this result was thought at one time to indicate that electron transfer proceeded by a different pathway, the terminal oxidase being a flavoprotein or perhaps an auto-oxidisable cytochrome *b*.

Spectroscopic studies of the cytochromes in the pupal tissues have shown that cytochrome $b_5$ and cytochrome oxidase are

indeed present during diapause, although in somewhat reduced amounts. But the most significant change is the virtual disappearance of cytochrome *c*[19].

The action of dinitrophenol on the diapause respiration is important in this context. Dinitrophenol is known to increase the turnover of respiratory carriers, probably by uncoupling oxidative phosphorylation from electron transfer, and so increasing the demand for oxygen. In the cecropia pupa dinitrophenol injections may cause a seventeen-fold rise in oxygen uptake. Moreover, the dinitrophenol-stimulated respiration is CO-sensitive, showing that dinitrophenol increases the saturation of cytochrome oxidase[20].

These results have led to the suggestion that CO- and CN-insensitivity arises as a result of the great excess of cytochrome oxidase in the diapausing tissues relative to cytochrome *c*. A high proportion of the enzyme is therefore unsaturated and can be immobilized with inhibitors without affecting electron transfer from cytochrome *c*. Thus cytochrome oxidase may well be the terminal oxidase in diapausing as well as in growing insects.

The cecropia pupa is a rather extreme example of the insensitivity often shown by dormant insects to respiratory inhibitors. Other species are much less resistant—the weevil *Sitona*, which hibernates as an adult, is an example[21]. Such differences are probably related to the amount of muscle present in the diapausing insect. In the cecropia pupa there is little of this tissue save for the small intersegmental muscles which serve to flex the abdominal segments. These muscles do in fact retain an intact cytochrome system, but their total volume is small relative to the pupa as a whole. Dormant larvae or adult insects are more mobile and retain a correspondingly large proportion of muscles. These of course contain the sarcosomes with which the cytochrome system is associated.

A very interesting finding which is still incompletely understood concerns the striking stimulation of respiration which takes place when a diapausing insect (for example, the cecropia

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pupa) is wounded or subjected to surgical operations. The effect is not localised but is due to increased oxygen consumption throughout the body. The stimulated respiration is certainly associated with the synthesis of cytochrome *c*, since the stimulated respiration is inhibited by CO. Nevertheless, it is significant that although the metabolism may be raised almost to the level found in the developing insect, no development in fact takes place, since there is no secretion from the prothoracic glands. The endocrine system is therefore the prime mover in the control of diapause.

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DISCUSSION

GALUN: Does Carrol Williams' experiment mean that *H. cecropia* does not need any gonadotropic hormone for egg maturation?

LEES: That is so. The control of egg development by hormone varies greatly in different insects. *Calliphora* requires material from the neurosecretory brain cells for egg development. Many require juvenile hormone from the *corpus allatum*, but *H. cecropia* pupae will develop viable eggs after removal of the *corpora allata*. The puzzling thing is that the *corpora allata* of male *H. cecropia* adults produce large quantities of hormone which the moth does not appear to require at all.

GALUN: Is it possible, in your experiment with aphids, to illuminate the internal organs directly rather than through the epidermis, and get the same effect?

LEES: It would be very difficult technically, since the aphids must be allowed to feed freely on the host plant after each cycle of illumination.

PENER: What is your opinion on Bodenstein's experiment on the disappearance of the thoracic gland after the last moult in the adult cockroach? But when the *corpora allata* had been extirpated the thoracic gland remained intact and extra moulting of the adult occurred.

LEES: As in other insects, attainment of the adult state in *Periplaneta* is accompanied by the degeneration of the thoracic glands; and this is the immediate reason for the cessation of moulting. Bodenstein has shown that, under experimental conditions, the thoracic glands can be caused to remain active in the adult, and the adult then moults. The necessary conditions for preserving the thoracic glands seem to be satisfied when the *corpus cardiacum* is active and the *corpus allatum* inactive.

SHULOV: I should like to refer to an experiment made on larvae of *Trogoderma granaria*. One set of larvae continued to moult over a period of eight months becoming gradually smaller until they became microscopic. The other set did not moult at

all. Yet both sets came from the same breed and no differences in external conditions seemed to exist.

LEES: I can think of no convincing explanation for the type of variation you mention. But I would like to call attention to the recent work of Burges on the same species. Larvae, which from their low respiratory rate would be judged to be in diapause, nevertheless still moult at intervals. In the process they often become smaller.

REINHOLD: Is there not a parallelism between the waking from diapause and the stimulation or fertilisation of sea-urchin eggs? The unfertilised eggs, for example, have a respiration not sensitive to cyanide, and become sensitive to cyanide on fertilisation.

LEES: Yes, I think you could call the unfertilised egg a diapausing egg.

MUEHSAM: You mentioned a case of the influence of neurosecretory brain cells on oviposition, where the cutting of the axons resulted in oviposition in an unfed adult. On which insect was this experiment done?

LEES: On *Oncopeltus*. Johansson has shown that in the fasting insect the brain inhibits the *corpus allatum* through the commissures. When these are cut, the inhibition ceases and egg-laying begins. There is, of course, much variation according to species. For example, Ellen Thomsen has shown that quite a different mechanism is in control of egg maturation in *Calliphora*.

HARPAZ: I would like to draw an analogy between the breaking of diapause in insects and dormancy in seeds, referring to the mechanism of mechanical injury. There is evidence from Theron that the diapausing codling moth larvae in a cocoon repair the cocoon even if the latter is torn several times, but finally the insect gives up, pupates and resumes its development. This could provide an analogy to mechanical injury in seeds.

LEES: These experiments were repeated by Andrewartha in Cambridge who did not succeed in breaking diapause by removing the cocoon. This was, of course, an English strain not a South African one, as in Theron's case.

HARPAZ: In experiments we have conducted, diapause was terminated by cold shock treatment, consisting of chilling to —10° followed by immediate transfer to 25°. This procedure probably has no ecological equivalent, as nowhere are such conditions extant.

LEES: I think I must disagree with you here. There are some investigators who believe that this finding does have an ecological application. These include Danilyevsky who found that in the moth *Saturnia pavonia* very low temperatures can terminate diapause; and Way has made similar observations on the fly *Leptohylemyia*. These authors point out that in continental regions of the north temperate zone, the warmest part of the winter comes first, really cold conditions being delayed until the early months of the year. It is possible that this final stimulus is required to complete the processes which result in the release from diapause.

HESTRIN: In view of the long time period which elapses until the effect of temperature change on the eggs becomes manifest, one might wonder whether the effect might not involve crystallisation or solubilisation of components present in the cytoplasm. Has it been possible to see alterations by microscopic examinations of the tissues?

LEES: Yes, cytological changes in the neurosecretory cells of a few diapausing insects have been described, but their significance is doubtful. So far, such changes have merely been taken as indicating that the particular cells are actually involved in diapause control.

# ENVIRONMENTAL FACTORS IN INTERRUPTION OF DEVELOPMENT OF ACRIDIDAE EGGS

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The ways in which Acrididae eggs may develop are manifold. The eggs of tropical and subtropical locusts *Schistocerca gregaria* (Forskål) and *Locusta migratoria migratorioides* (R. and F.) usually develop continuously and hatch within a comparatively short period. Some others also develop continuously under field conditions, but there may be a slow development at lower temperatures, as in the case of *Pareuprepocnemis syriacus* (Table I).

There are many other types of development which include interruption at various stages of the embryonic development, such interruptions being caused by intrinsic as well as extrinsic factors, *e.g.* temperature and humidity or both these factors combined.

Slifer[1], Steele[2], Bodenheimer and Shulov[3] and Shulov and Pener[4] provided the basis for the study of embryonic development in some Acrididae by describing morphological stages in connection with the time of their appearance (Fig. 1).

Shulov[5] classified interruptions in embryonic development of Acrididae according to four types.

The first may occur in the initial period after oviposition, when the egg is dormant and no embryonic germ-band appears during a period of two to four weeks. This type of pause appears in *Dociostaurus maroccanus*[3], *Austroicetes cruciata*[2] and in *Tmethis pulchripennis asiaticus*[6]. In *Dociostaurus*, the eggs during this period are not influenced by temperature. The water content of the eggs of *Dociostaurus* and *Tmethis* is at equilibrium or they lose some water: the addition of water during this period kills the eggs of both species.

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TABLE I

THE DURATION OF NONINTERRUPTED DEVELOPMENT OF EGGS IN SOME ACRIDIDAE AT 27° ± 1°

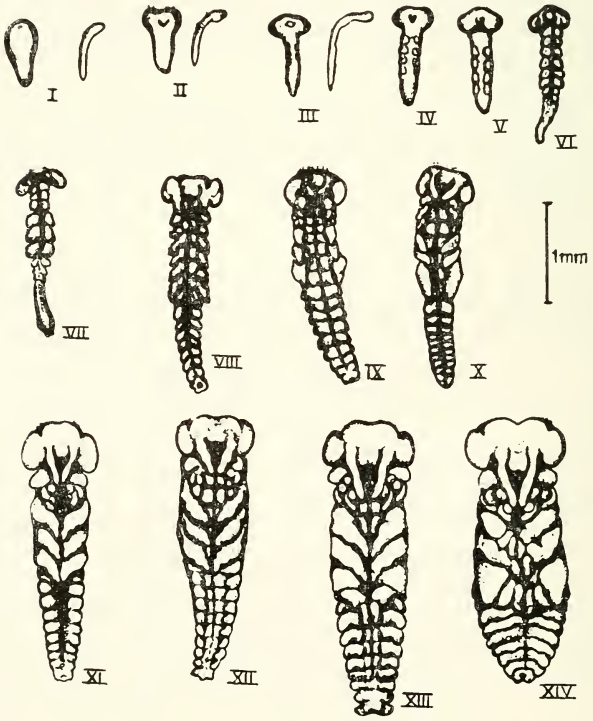
| *Species* | *Duration of development in days* | | *Temperature °C* | *Reference* |
|---|---|---|---|---|
| | *Mean* | *Range (Min-Max)* | | |
| *Schistocerca gregaria* (Forskål) | 17.63 | 15–21 | 27.0 | Shulov and Pener[11] |
| *Schistocerca gregaria* (Forskål) | 16.0 | — | 26.7 | Hamilton[18] |
| *Locusta migratoria migratorioides* (R. and F.) | 16–17 | 14–18 | 27.0 | Shulov and Pener[4] |
| *Locusta migratoria migratorioides* (R. and F.) | 15.0 | — | 26.7 | Hamilton[18] |
| *Nomadacris septemfasciata* (Serv.)* | 32.0 | 27–40 | 27.0 | Shulov and Pener (manuscript in preparation) |
| *Acrotylus insubricus* (Scopoli)* | 23.4 | 22–26 | 27.0 | Shulov, Pener and Slavin (manuscript in preparation) |
| *Pareuprepochnemis syriacus* (Br.)* | 43.6 | 42–57 | 27.0 | Shulov, Pener and Slavin (manuscript in preparation) |
| *Anacridium aegyptium* (L.)* | 35.2 | 33–46 | 27.0 | Shulov and Pener (manuscript in preparation) |

* Preliminary figures.

The second type includes those eggs which show a slow development during anatrepsis, influenced to a certain degree by rise in temperature, as in the cases of *Locusta migratoria*[7], *Tmethis pulchripennis asiaticus*[6] and *Calliptamus palaestinensis*[8]. In *Dociostaurus*[3] the influence of temperature at this stage is not significant and this period may last up to five months.

The eggs during this period are not affected by addition of water in *Calliptamus* and *Locusta*, but are spoiled in the case of *Dociostaurus* and *Tmethis*.

The third type occurs at the end of anatrepsis and includes all forms of diapause which cannot be broken by changes of humidity or temperature. During this period the embryo is



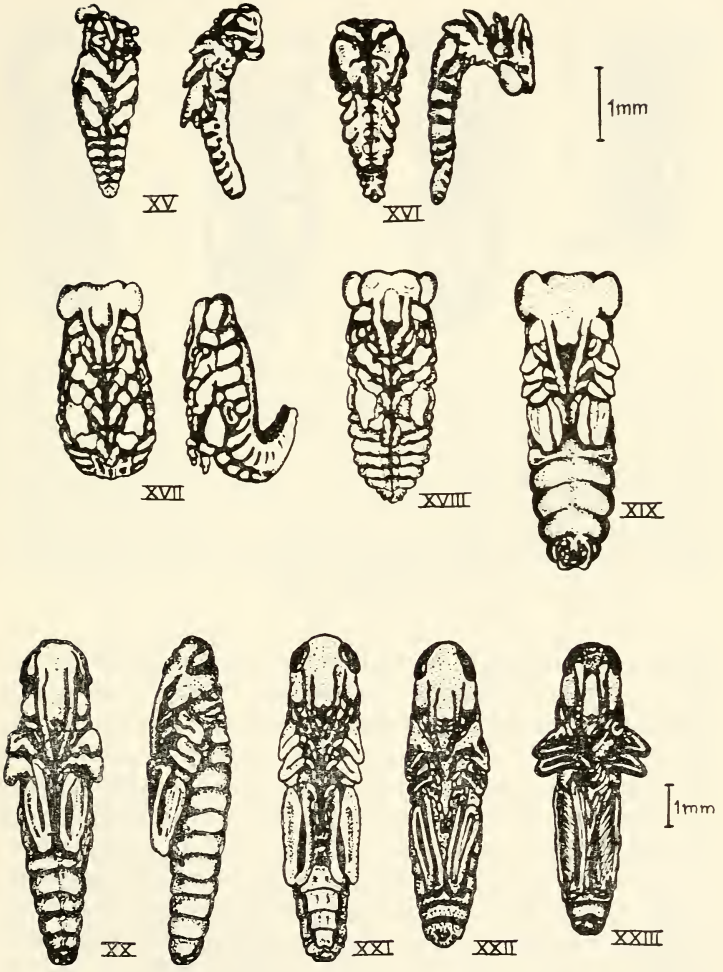



Fig. 1. The embryonic stages of *Locusta migratoria migratorioides* (R. and F.) (according to Shulov and Pener[4]). The embryos are shown on three different scales, from the ventral side. Lateral views of some of the stages are also included.

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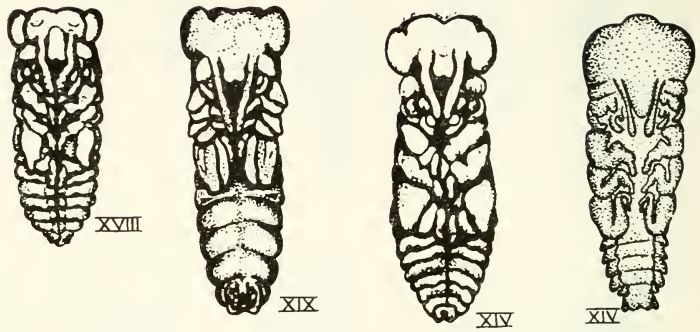


Fig. 2. Embryos of *Locusta migratoria migratorioides* (R. and F.) (stages XIV, XVIII, XIX) and of *Dociostaurus maroccanus* Thnb. (stage XIV, in the right corner (punctated)). (*Locusta* embryos according to Shulov and Pener[4], *Dociostaurus* embryo according to Bodenheimer and Shulov[3]). The embryos of stages XVIII and XIX of *Locusta* are on the same scale, but that of stage XIV is magnified to match the size of embryo of *Dociostaurus maroccanus* shown. The thoracal appendages of the *Dociostaurus* embryo show more advanced differentiation, in comparison to the *Locusta* embryo of the same stage.

ready morphologically for katatrepsis, but not physiologically. This type of interruption is known in *Dociostaurus*[3] and in diapause-bound eggs of *Locustana pardalina*[9]. The diapause may last from two to several weeks. It seems that in *Dociostaurus* this period is not influenced by temperature or humidity, but in *Locustana* the addition of water at this time has apparently an adverse effect, *i.e.* causes prolongation of the diapause.

The fourth type of interruption comprises embryos at the end of anatrepsis which will resume development if provided with a sufficient amount of water. The interruption in such cases may persist for various lengths of time, ranging from five months in the eggs of *Calliptamus palaestinensis*[8] to more than three years for the eggs of *Locustana pardalina*[10] (Table II).

The species mentioned in Table II (part A) are typical for arid climates. Their eggs are laid mostly in dry soil. Such species

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TABLE II

MAXIMAL EXTENT OF THE TYPE FOUR DIAPAUSE IN THE EGGS OF SOME ACRIDIDAE

| *Species* | *A. Duration of diapause** | | *Reference* |
|---|---|---|---|
| *Locustana pardalina* | Three and a half years | | Faure [10] |
| *Dociostaurus maroccanus* | Seven months | | Bodenheimer and Shulov [3] |
| *Prionosthenus galericulatus* | Seven months (room temperature) | | Shulov and Pener (manuscript in preparation) |
| *Calliptamus palaestinensis* | Five months (room temperature) | | Pener and Shulov [8] |
| | *B. Induced diapause in laboratory at 27°* | | |
| *Schistocerca gregaria* | 98 days in 100% relative humidity | At least 21 days in 60% relative humidity | Shulov and Pener [11] |
| *Nomadacris septemfasciata* | 170 days in 100% relative humidity | At least 28 days in 60% relative humidity | Shulov and Pener (manuscript in preparation) |

* Maximum observed.

achieve a morphologically more advanced state at stage XIV, than species which do not as a rule show any diapause. We have been able to demonstrate that in *Schistocerca gregaria*[11] as well as in *Locusta migratoria migratorioides* and *Nomadacris septemfasciata* (Shulov and Pener, manuscript in preparation), eggs deprived of the full quantity of water needed for completing development reach stage XIV only, and then enter into a developmental pause which may be broken in the same way as in the above group, *i.e.* by addition of water (see Table II, part B). However, the morphological stage in which this interruption occurs is significantly less differentiated than is the stage XIV of eggs which interrupt their development at the fourth type of diapause almost always under natural conditions (Table IIA)[4].

Two further types of interruption may be added to the four types already mentioned.

One has been observed in *Melanoplus differentialis*, where the break of diapause is connected as a rule with low temperatures[1] under natural conditions. This type of interruption of development occurs at the end of anatrepsis. Resumption of development generally takes place when the eggs are transferred from low temperature conditions to higher ones, but it is still possible that the upper limit of the lower temperature range responsible for the break of diapause merges with the lower limit of that temperature range which allows renewal of slow development after the end of the diapause.

This type of interruption is manifested in the northern form of *Locusta migratoria*[12], in *Calliptamus palaestinensis*[8] and possibly in *Austroicetes cruciata*[2] and *Chorthippus brunneus*[13]. It is typical of temperate regions.

A sixth type of interruption of development has been stressed by Slifer[14] regarding those Acrididae which enter a diapause shortly before hatching. This type of diapause appears in some species of the genus *Melanoplus* such as *M. bivittatus* and its duration seems to be shortened by the influence of low temperatures, near 5°[15].

Another group seems to exist in which the development of

eggs is interrupted by temperatures below their developmental threshold[16]. We are unable to classify them into the above proposed scheme, as the stage or stages at which the embryonic development is interrupted are not known to us.

Apart from environmental factors such as temperature and humidity, it seems that the structure of the egg envelope, and especially that of the hydropyle, is of essential importance at least in *Melanoplus differentialis* type of eggs. Weathering and other changes in these structures play an important role in breaking of diapause, according to Slifer[14] and Lees[17].

The intrinsic factors connected with appearance and breaking of diapause in Acrididae are still unknown. It is quite possible, however, that their influence is not less important than that of the extrinsic influences.

It is unnecessary to stress the survival effect of diapause and its importance in the adaption of the species, *cf.* Acrididae, to their environment. We should like only to point out that, in the case of *Calliptamus palaestinensis*, in which interruptions in development of the fourth and fifth types combined take place, we have been able to show recently[8] how well the way of the development fits the climatic conditions of the geographic region of its distribution. The eggs of *Calliptamus palaestinensis* are laid in autumn and develop slowly in moist as well as in dry soil. For their successful development both cold temperatures of the local winter and the rains occurring late in spring, are essential.

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## DISCUSSION

Lees: I would like to ask Dr. Shulov whether he thinks that Slifer's observation on the eggs of *Melanoplus differentialis* are applicable to his species. In *Melanoplus* Slifer demonstrated a physical barrier to the uptake of water, which is probably a waxy substance removable by mild solvents, such as medicinal paraffin. At the same time as the egg goes into diapause, this waxy layer is deposited on the hydropyle and development ceases. When the layer is dissolved and water absorbed, development continues.

Shulov: We have been unable to find a similar mechanism regarding the formation of the hydropyle in *Dociostaurus* eggs, which under our conditions have a natural diapause. We find here another kind of grasshopper, *Tmethys*, which develops without any addition of water. Its hydropyle does not show any significant change during development. Therefore it appears to me that the physical change around the hydropyle is generally less important.

Lees: Do you think that the low temperature acts upon the embryo or upon the cells underlying the hydropyle?

Shulov: I could not say, since we failed to isolate viable embryos.

Galun: Did you ever try to break the shell mechanically, in the case where the composition of the waterproofing material is unknown?

SHULOV: We tried it on *Dociostaurus* and found that pierced eggs imbibed water but the embryo did not develop and did not turn around.

PENER: I would like to refer to my experiments on *Calliptamus palaestinensis*. The diapause eggs of this grasshopper were treated by Slifer's method, some of them with mineral (paraffin) oil and others with xylol, but development was not resumed. The eggs imbibed some water, however, a distinct imbibition was observed also in the untreated eggs during diapause. Some months later the treated and untreated eggs were transferred to low temperature and the majority resumed development.

# SOME OBSERVATIONS ON THE ROLE OF DIAPAUSE IN THE PHENOLOGY OF INSECTS IN SEMI-ARID ZONES

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A subtropical climate, in particular a Mediterranean climate, is characterized by a long dry summer and a mild rainy winter. Such an annual cycle induces in many insects a summer-heat and/or drought diapause, and in a smaller number of cases a winter-cold diapause. The latter is predominant in Central and Western Europe, and its manifold aspects have already been extensively studied there, so much so that hibernation and diapause are often used as synonyms. On the other hand, heat and drought quiescence of aestivating insects are phenomena which have been much less considered, particularly heat-diapause in its strictest sense. Incidentally, diapause, aestivation and quiescence are not wholly synonymous, as Lees[1] has pointed out very clearly. Reference at this stage should be made to Rivnay's paper[2], which was the first appraisal of the problem in this region.

Let us take as an example the codling moth (*Cydia pomonella* L.). It is essentially univoltine in Northern France and England, where over 90% of the fully-fed larvae enter diapause by the end of summer and pupate only the following spring. In the South of France, however, where the summer is warmer and development is faster, about 70% of the larvae pupate before entering diapause and give rise to a second generation[3]. In Israel, with an even warmer summer, where the flight of the first generation takes place in April and May (as compared with June and July in Western Europe, including Southern France) there are even three generations a year[4] with diapause setting in as from mid-July and onwards. This situation can be satis-

factorily explained on the basis of Dickson's studies on the photoperiodicity of this moth, which conclusively demonstrated that the codling moth responds to a critical day-length of 15 h, while with 12 h of daily illumination almost all larvae enter diapause[5]. It should be pointed out that in all the countries mentioned previously the codling moth caterpillars enter diapause as from mid-July at the earliest, *i.e.* at a period of shortening day-light hours.

However, in the vicinity of Baghdad, where conditions would be expected to be favourable for an even greater number of generations than in Israel, it was observed by the late Prof. Bodenheimer that the larvae entered diapause as early as in the middle of May, and thus gave rise to no more than one annual generation. As diapause occurs there during a period of increasing day-length (the average number of day-light hours during May in Baghdad is 13 h 35 min), the diapause-inducing factor in Iraq should be sought elsewhere than in the photoperiod effect. A simple experiment carried out in our laboratory at Rehovot[6] revealed that larvae hatched during the first half of April, which after penetrating fruits were transferred to an incubator at 27°, all went into diapause, regardless of host fruit variety. The same thing happened when the larvae were exposed to 27° following some 17 days of natural outdoor development, which constitutes about three quarters of the total duration of larval development. On the other hand, when fully grown larvae were collected outdoors in May and exposed to the same 27° constant temperature, only 42% went into diapause and the rest pupated and emerged the same summer. Simultaneously with this experiment, when another group of larvae, hatched in May, were exposed to a temperature of 23°, all pupated at the expected time. The average monthly temperatures in Baghdad[7] are 22° in April, 28° in May and 32° in June, whereas in Rehovot the monthly mean temperature reaches 26.5° only in August, which is the time our codling moth begins its diapause. Another conclusion to be drawn from this experiment is that the stage of development sensitive to high tempera-

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ture as a diapause-inducing factor is the last quarter of the duration of larval development, in other words the last instar.

Undoubtedly diapause in the codling moth in general is governed by a variety of factors, *i.e.* photoperiod, temperature, diet and perhaps others yet unknown, each one of which may become predominant according to the prevailing circumstances.

Perhaps a more striking example is the phenology of the onion fly (*Hylemyia antiqua* Meigen) in Israel as recently reported by Yathom[8]. The same species is a well-known pest in Europe and North America where according to the phenology of its liliaceous host plants of the genus *Allium*, it enters the pupal diapause during August and September, and development is only resumed after winter, in May[9]. In Israel, in contrast, *Allium* plants under natural conditions germinate or sprout only after the first winter rains in November-December, and grow till the end of the rainy winter season, at the end of which they become dormant for the dry summer season. In close synchronization with this cycle the onion fly emerges in November and breeds one generation up to January. The majority of the offspring of the following generation enters the pupal diapause in March. This takes them through the dry period of absence of fresh *Allium* growth till November or December, depending on the first rain. A similar instance is that of the aphid-eating syrphid fly *Epistrophe balteata* de Geer, which in Switzerland, for example, diapauses as an adult during winter, when aphids are very rare[10], whereas the same species in Israel raises several generations during winter and spring, when aphids are abundant, and is dormant during summer when its prey becomes extremely scarce[11].

One cannot assume that the factors which induce winter diapause in the onion or syrphid fly in the temperate zone are precisely the same ones that cause the same species to aestivate in a subtropical region, though the reason for arresting development is the same, namely, to survive a period of absence or shortage of food. This must lead one to suspect that the changes which take place within the host plant in anticipation of an

impending crisis affecting its development (oncoming winter cold in the temperate zones, or the approach of dry summer in the semi-arid zones), must be communicated to the insects consuming those plants. In other words, either diapause-inducing or development-inhibiting substances are to be sought in the diet of insects, especially those exhibiting facultative diapause. This should be a mechanism somewhat similar to that of the response to the host in parasitic insects, although Dr. Lees will probably argue that the latter is a case of quiescence rather than of strict diapause.

Before concluding I should like to raise briefly one more point. One might be highly tempted to try to dismiss the whole issue by the trite argument that we are dealing with quite distinct geographical or ecological races of the species; in the case of the codling moth different names have even been given, *viz. Putaminana* Stdgr. for the Near Eastern race. It might in turn be argued that each of these races hereditarily responds to a certain consistent environmental agency, whether as regards the onset of diapause or its termination, and not to alternative agencies, according to the different environments. Allow me to avert these temptations by quoting a few lines from an article written by Bodenheimer and Vermes[1,2] some three years ago: 'Diapause depends upon the interplay of heredity and environment. Hence, no general genetical solution of the problem of diapause determination can possibly be given. Once the one, once the other factorial group may be the main inducing factor. Yet even under apparently similar environmental conditions during the critical period or in populations of an apparently higher homogeneous genetical constitution may result as different phenotypical manifestations.'

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DISCUSSION

Lees: I do not think it profitable to argue whether it is an example of diapause or quiescence; everybody should be allowed to make up his own mind on this point. My question refers to Gambaro's work in Italy. She believes that the maturity and the variety of the apple has a considerable influence on diapause in the codling moth. Is your Baghdad race affected similarly?

Harpaz: There is no synchronisation between the phenology of our apple trees and that of the codling moth. When the insect emerges before the fruit is available, it finishes its larval development entirely on leaves.

Hestrin: You have suggested that the induction of diapause prior to nutritional deficiency is a useful characteristic; but how does selective pressure in evolution produce an anticipatory mechanism?

Harpaz: This was only a suggestion. I feel it must be cumulative over a period of time, you cannot induce diapause by shock treatment.

Lees: I do not see any great difficulty here. If natural selection weeds out the animals which do not enter diapause, 'anticipatory' mechanisms would surely be evolved rather rapidly.

# ECOLOGICAL PROBLEMS OF SEED DORMANCY

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Dormancy in seeds is a misleading term. A seed which does not germinate when placed in moist soil is just as dormant as a plant which does not flower during long days because it requires short days for flowering. The only difference between the two is that in the case of the seed we are dealing with initiation of growth while in the other we are dealing with initiation of flowering. In both cases the process of initiation is triggered and controlled by environmental signals, and may be said to be regulated by the environment[1]. Some seeds require a period of so-called 'after ripening' under a specific range of environmental conditions before they are ready to germinate[2–4], and analogously many plants require a certain period of vegetative growth under a specific range of environmental conditions before they achieve 'readiness to flower'. Also, many seeds are known to become less dormant as they age, insofar as they germinate within a wider range of environmental conditions[5, 6], and analogously many plants which require a certain photoperiod or vernalization for flowering may eventually flower without them when they are sufficiently old.

If we accept this analogy we may approach the question of the significance of these phenomena in the existence of the species. The common denominator for these phenomena is the existence of a biological system which permits a choice between starting a new developmental phase and continuing in the previous one. It is logical to assume that such systems have evolved as a result of their being of survival value to the species. Presumably a photoperiod-sensitive plant utilizes the most dependable time-telling device, the astronomical clock, to choose the time of flowering suitable for that particular species in its particular habitat to carry out to completion one essential part of its

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program of survival, namely formation of viable seed. The question under discussion here is if and to what extent regulation of germination performs a similar function in another part of the program of survival of the species, namely establishment of the seedlings in a suitable environment at a suitable time.

Several germination regulating mechanisms have been identified in seeds, for example control of water entry into seed, control of the gas exchange between the environment and the embryo, germination inhibitors, control by specific temperature response, control by light response. Some of these mechanisms may indeed be visualized as bearing ecological significance. Thus, the control of water entry into the seed, which is achieved by the presence of a water-impermeable layer surrounding the embryo[2, 3, 7], may operate in two ways. The impermeability may disappear from a fraction of the seed crop at a time over a long period[8, 9], or it may disappear simultaneously from almost the entire population through the action of some environmental factor (frost[10], heat[11], microbial activity in the soil[12] or in the stomach of ruminants[13, 14], or at specific relative humidities[15] or by action of fire[16]). In the former case, the repeated attempts at establishment compensate for the haphazardness in choice of environment. In the latter case, specific environments would be favoured.

Regulation by germination inhibitors has some clear ecological implications, in that certain environments are excluded and others are favoured[17]. Thus, inhibitors in pulp and juice of fleshy fruits exclude precocious germination inside the fruit[18]; water-soluble inhibitors in dispersal units of desert plants may act as rain-gauges in preventing germination until a certain critical amount of rain has fallen[19–23]; inhibitors may also aid in preventing over-population by seedlings (whether of the same or other species)[24].

Control by specific temperature also has some obvious ecological implications. The simplest of these are the requirements for specific ranges of more or less constant temperaf tures[25, 26]. A more sophisticated control is found in seeds o-

temperate-zone plants, which require 'stratification', namely, pretreatment of the moist seed by cold[3, 4]. This restricts germination not only to post-winter conditions, thus minimizing the dangers of frost killing, but also to regions with cold winters, which most of these plants require for other developmental phases (*e.g.* breaking of bud-dormancy, flowering). A similar argument may be postulated for seeds the germination of which is optimal at diurnally fluctuating temperatures[20, 27–29, 30–33]. It is likely that such a requirement confines germination to regions, seasons and soil depths where such fluctuations occur[1, 20, 34].

Control by specific light requirements may operate in germination in much the same way as it does in flowering, by providing the process with a celestial clock. Short day seeds and long day seeds have been described[35, 36]. In addition, since the embryo is heterotrophic, light-inhibited seeds may also be considered short day seeds, while seeds which require even a flash of light are comparable to long day seeds. Seeds of many hygrophytes require light for germination[37–39]. Here it is likely that the requirement places a limit to the water-depth at which germination will occur[1]. A similar mechanism may be operative in plants which form forest undergrowth.

Several problems present themselves in studies of this nature. Two of these stand out because of their importance and the danger of ignoring them. One is the fact that the vast majority of our information on germination has been obtained by experiments on or between filter papers, in Petri dishes or germinators and using distilled water. Not only may some of the data be a result of these experimental artifacts, but data may have been overlooked. A case in point is that of lettuce seeds which at 20° appear light-insensitive when germinated in water, but require light when germinated in solutions above a given osmotic value[40]. The other important consideration is fundamental to ecology. To what extent are the ecological implications more than rationalization of phenomena which have little, if anything, to do with the actual situation? It is dangerous as it is

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tempting to ascribe profound meanings to biological phenomena. Yet if such meanings actually exist their elucidation may be of the utmost importance. With regard to germination, we can only point to the general fact that cultivated species, in which dormancy has been selected against, require constant reseeding, while most wild species, in which regulated germination has been naturally selected for, are hard to eradicate by any single treatment. This fact is suggestive, but experimental techniques have to be devised for testing the hypothetical ecological implications of the so-called dormancy in seeds.

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## DISCUSSION

Lees: If the low temperature requirement for seeds is a kind of area restrictive device, I cannot see its value from the evolutionary point of view. Why should the evolution not be such that, while such a device is present in cold climates, the device gradually disappears when the plant moves to a warmer climate. This is what happens in insects.

Koller: Certain varieties growing in moist regions require much higher rainfall to wash away inhibitors of germination than other varieties of the same plant growing in deserts. This may be a partial answer to your question in a parallel case.

Mayer: What is the ecological or survival value of the Red–Far Red mechanism in germination and in flowering?

Koller: The Red–Far Red system is just one part of the intricate 'biological clock' by which the organism recognizes the seasons.

KEYNAN: Is this light sensitivity not a means for the seed to know that it is at the right depth for germination?

KOLLER: This is unlikely, as lettuce seeds planted in the soil are no longer light sensitive.

NACHMONY: The temperature range may be another explanation. Wareing found that the germination of *Betula* seeds at 15° can be induced by photoperiodic treatment, while at 26° a flash of light was sufficient to initiate germination.

KOLLER: In our laboratory germination was independent of light at 20° or below.

LEES: Germination in the dark might be equivalent to germination in continuous light.

GALUN: What is the ecological value of low oxygen tension?

KOLLER: Marsh plants germinate only under marsh conditions. I would like to remark that we cannot generalize about 'normal' conditions, since every variety has its conditions for germination, and the dormancy of the seed may be dependent on the availability of these specific conditions.

# ROLE OF PLANT HORMONES IN DORMANCY OF SEEDS

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The importance ascribed to plant hormones in controlling various functions and processes of plant growth and behaviour is increasing rapidly.

The first group of plant hormones isolated and identified was that of the auxins. These were discovered in connection with studies on stem elongation, and extension growth is still the basis of some bioassay methods for this group of hormones. They are sometimes designated as plant growth substances[1, 2]. In this group are classified all the substances causing extension growth and having in their structure an unsaturated aromatic nucleus and a side chain terminating in a carboxyl group or one that is easily oxidized to a carboxyl. The best known representative of this group is 3-indolyacetic acid (IAA).

As research was extended, it was proved that the same substances actually affect numerous and very variable aspects of plant development, such as root initiation, cambial activity, apical dominance, leaf abscission, fruit growth and many others[1, 3]. For many years the auxins were the only identified group of hormones; other hormones such as the 'calins' and the 'florigens' were postulated, but not isolated or identified.

Recently, two new groups of substances were isolated and their effect on plant development allows us to include them among the plant hormones; these are the gibberellins and the kinins. The gibberellins were first isolated as products of fungal metabolism[4, 5], and kinetin, the best known compound among the kinins, was artificially produced from nucleic acids[6]. In due course it was shown that higher plants apparently contain substances of similar structure and activity.

Evidence will be brought here to show that the three groups

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of hormones have some role in regulating dormancy in seeds. There are also other substances found in the seeds but not as yet identified, which take part in regulating the dormancy phenomenon and germination processes. These substances are either germination inhibitors or germination promoters, and they are the products of normal metabolic processes in the ripening and in the germinating seed. Similar substances regulate the dormancy and sprouting of buds.

As has already been explained in previous lectures, various dormant seeds will not germinate unless their dormancy is broken by special temperature or light treatment. In some cases such treatment may be avoided and the seeds may be induced to germinate by treating them with plant hormones. Such seeds, for example, are lettuce seeds (*Lactuca sativa* L.) variety Grand Rapids. These seeds, for several years after harvesting, will not germinate in the range of temperatures of 23–28° unless illuminated, when fully imbibed, for a certain period of time with red or white light. This light requirement diminishes with increasing period of storage, and five to six years after harvesting the seeds will germinate in the dark to the extent of 80% or more. Irradiation with Far Red reverses the effect of red and usually also lowers the percentage of germination in the dark.

The red light treatment breaks the dormancy of the seeds and forces them to germinate. A similar effect may be achieved by soaking the seeds in a solution of gibberellin[7, 8, 9]. The mechanisms of the two treatments are not identical as the effect of both of them is additive (Table I) and the Far Red completely reverses the red effect but only partially the gibberellin effect[7].

Besides in lettuce, gibberellin has been shown to be effective in breaking the dormancy of *Arabidopsis*[10], *Kalanchos*[11] and many other species[12, 13].

A closer interaction between the effects of light and a plant hormone in breaking dormancy was shown for kinetin[14, 15]. Kinetin greatly increases the sensitivity of the seeds to red light. In the presence of kinetin, extremely small amounts of light 720 ft.c.s. (foot-candle-seconds) are sufficient to bring about maximal

TABLE I

THE EFFECT OF LIGHT AND GIBBERELLIN TREATMENTS ON THE GERMINATION OF LETTUCE SEEDS

Results given as percent germination. The red illumination was given after two hours' imbibition. The Far Red was given either immediately after the Red or, when given alone, after 30 min of imbibition. The concentration of the gibberellic acid was $2.9 \times 10^{-5}$ *M*.

| | *Water* | *Gibberellic acid* |
|---|---|---|
| Dark | 12 | 39 |
| Red | 44 | 66 |
| Far Red | 5 | 25 |
| Red and Far Red | 11 | 35 |

germination, while in the absence of kinetin 3600 ft.c.s. are necessary to bring about the same result[15].

In all these cases it is not clear what are the actual mechanisms that are affected either by light treatment or by the hormones.

The possibility that IAA may play a part in the germination of seeds has been considered for many years, but the results of the experimental work were rather contradictory. Some suggested an inhibition of germination by excess of auxins present in the seeds or in the fruits[16, 17], while others suggested stimulation[18, 19]. Söding and Wagner[36] tried to settle this controversy by suggesting that IAA stimulates the germination of dormant seeds but does not affect the germination of non-dormant ones. They tried unsuccessfully to test this hypothesis on Poa seeds but the idea proved to be correct for lettuce seeds[19–21, 36]. The more dormant the seeds were, and the lower the temperature during germination, the more effective was IAA in breaking the dormancy and in stimulating germination.

The next step was, therefore, to find out whether the seeds contain any endogenous IAA and whether its amount changes during germination. Dry seeds and seedlings of lettuce of varying

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ages were extracted and the extracts fractionated and separated with the aid of paper chromatography using the conventional methods for IAA isolation[22]. The various parts of the chromatogram were assayed using the extension growth bioassay. The results are summarized in Fig. 1.

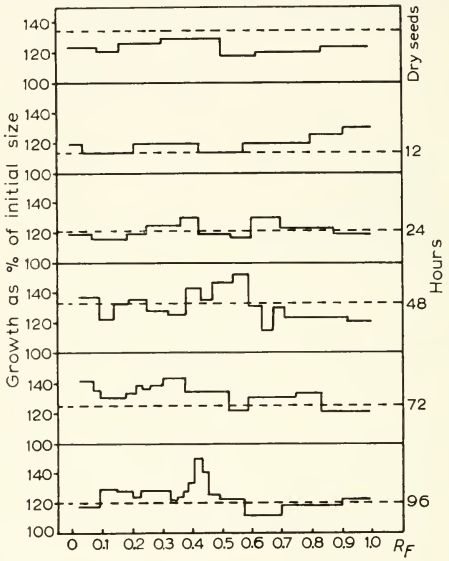


Fig. 1. Histograms showing the chromatography separation of acid growth-active substances in lettuce seeds germinated for various lengths of time. The biossay used was the oat coleoptile extension growth test. The broken line gives the growth of the controls[22].

It is evident that dry seeds do not contain any IAA or any other acid growth promoter. They do contain growth inhibitors. IAA appears between $R_F$ 0.35–0.45, when the seedling already shows appreciable development.

Parallel to our work on the relation between growth promoting and growth inhibiting substances and dormancy, other groups have been engaged on the same problem.

Varga and her associates[27] and also Hemberg[23–25], are mainly interested in the mechanism regulating the dormancy of potato tubers, while Wareing and his group[29] are interested mainly in the dormancy of seeds and buds of woody plants. These are the principal groups working on the subject but there are many other investigators working in this field.

In all these investigations it very soon became clear that it is not possible to draw any definite conclusion, regarding the germination of seeds or the sprouting of buds, while using the extension growth bioassay. Special bioassay tests were, therefore, devised for every case.

Hemberg[23–25] ascribed the dormancy of the potato tubers to a growth inhibitor present in the potato peel. Blommaert[26] and Varga and Ferenczy[27] isolated this inhibitor and showed that its amount decreases as the tubers emerge from dormancy. But when this inhibitor was tested in a potato sprouting test[28], it was shown to have no effect.

More convincing results were achieved by Wareing and his group[29]. They showed that the dormancy of *Xanthium* seeds is to a large extent due to the presence of an inhibitor which prevents the growth of the embryo. Germination proceeds only after this inhibitor is either leached out or oxidized inside the seed. They have also shown that the dormancy of *Fraxinus* seeds[30] is regulated by an interplay between growth inhibitors and germination stimulators. The inhibitors present in the endosperm and the embryo itself, are probably the main cause of the dormancy phenomenon. During stratification there is no change in the inhibitor's content, but a germination stimulator is formed which counteracts the effect of the growth inhibitor and brings about the breaking of dormancy, thus enabling germination (Fig. 2).

Similar investigations were carried out in our laboratory with lettuce seeds[31]. The same light-sensitive variety (Grand Rapids) was used. The seeds were imbibed in water, in the germination inhibitor—coumarin, or in the germination stimulator—thiourea. Germination inhibitors and germination stimulators were followed up in all the three series (Fig. 3). It is evident that

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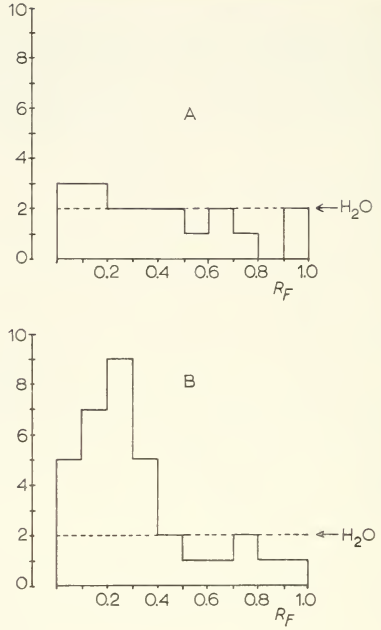


Fig. 2. Histograms showing the chromatographic separation of unchilled and chilled *Fraxinus* seeds. The bioassay used was *Fraxinus* embryo germination test. The broken line gives the water controls [30].

dry lettuce seeds and seeds imbibed in water in the dark contain germination inhibitors. On imbibition in water, the acid inhibitors disappear just before the actual germination begins, the neutral inhibitors being still present. This may explain the low germination percentage in the dark. On imbibition in coumarin, coumarin accumulates in the seeds[32] and apparently blocks germination. Comparison of the various histograms suggests that some of the natural inhibitors may also be coumarin derivatives. In another case[33] a natural germination inhibitor present in the seeds of *Trigonella arabica* was identified as coumarin.

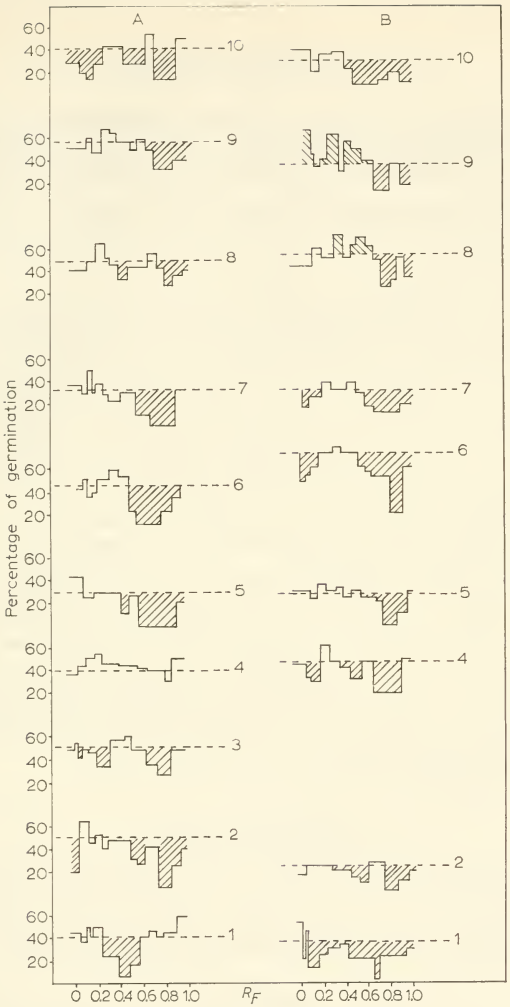


Fig. 3. Histograms showing the chromatographic separation of substances affecting germination extracted from lettuce seeds germinated in water, in coumarin or in thiourea.

A — acidic extracts; B — neutral extracts; 1 — dry seeds; 2 — seeds imbibed for 2 h in water in the dark; 3 — seeds imbibed for 4 h in water in the dark; 4 — seeds imbibed for 12 h in water in the dark; 5 — coumarin control; 6 — seeds imbibed for 2 h in coumarin; 7 — seeds imbibed for 12 h in coumarin; 8 — thiourea control; 9 — seeds imbibed for 2 h in thiourea; 10 — seeds imbibed for 12 h in thiourea. The broken lines give the water controls [34].

Thiourea, a germination stimulator, apparently changes the ratio between the various natural substances affecting germination, as it induces the formation of germination stimulators. This apparently occurs during the initial phases of imbibition. This change may initiate a chain of reactions eventually leading to germination.

To sum up the facts presented here, it seems that the plant hormones do affect germination, perhaps by changing the initial ratio between the natural germination or growth stimulators and inhibitors. This idea is supported by the facts that gibberellin counteracts the inhibitory effect of coumarin[34] and that lettuce seeds do contain appreciable amounts of gibberellin-like substances[35]. The natural inhibitors and stimulators are as yet not identified and we do not know whether they may be classified as hormones. A more complete study is required for the identification of these substances and for the evaluation of their changes under various treatments affecting germination. Although it seems possible that the factors involved in dormancy exercise their effect through the internal equilibrium of such hormones, it is as yet by no means understood which are the basic reactions affected by the hormones and it is as yet not certain that this is the only mechanism involved.

ACKNOWLEDGEMENTS

Our thanks are due to Prof. P. F. Wareing and to *Nature* for their permission for the use of Fig. 2 and to Dr. S. Blumenthal-Goldschmidt for her permission for the use of Fig. 3.

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DISCUSSION

KEYNAN: You mentioned, that by using a different part of the light spectrum you can 'unswitch' the stimulation of germination by red light. However, after about two hours this 'unswitching' is no longer possible and germination proceeds. Is there any visible or measurable change during these two hours?

POLJAKOFF-MAYBER: Our methods are not sufficiently refined to allow an answer to your question.

GOLDWASSER: What is the range of concentrations at which these inhibitors and stimulators act, and what quantities do you succeed in isolating?

POLJAKOFF-MAYBER: While we can extract quantities sufficient to obtain the necessary effects, these quantities are not sufficient for measuring purposes.

GOLDWASSER: Well, in that case you ought to be able to measure the changes occurring in the seeds indicated by Dr. Keynan.

POLJAKOFF-MAYBER: We hope to succeed in doing so, once we have refined our methods sufficiently.

# DORMANCY AND DORMANCY BREAKING IN SEEDS

S. KLEIN

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It would be very helpful if one could start a lecture like this with a clear and physiologically valid definition of what is meant by dormancy. The fact that it was thought desirable to compare this phenomenon in various groups of organism shows that we are still far from a general formula. In the broadest sense, dormancy in seeds means the cessation of growth in the embryo without the latter losing its viability for a prolonged time. The embryo may or may not be enclosed in its natural envelopes. These may consist of an endosperm, a seedcoat and other surrounding layers, which actually do not belong to the 'seed' proper. Since they may all influence the behaviour of the embryo it is more appropriate to talk about dormancy in 'dispersal units' than in seeds[1].

It is evident that such a period of dormancy is of extreme importance in the life of the plant. It allows effective dispersal of the young plants both in space and time, and allows the embryos, which are extremely sensitive in the active state, to overcome conditions unfavourable to development and growth.

It is also evident that dormancy is closely related to the subsequent renewal of growth. The processes which make this transition possible are therefore of importance in the understanding of dormancy, and we shall be concerned principally with changes that occur during dormancy breaking and with differences before and after, *i.e.* between seeds before and during germination.

This introduces another difficulty, namely, to define what is meant by germination. In order to renew its normal activities, the dormant seed has to be exposed to certain external conditions, *e.g.*, a certain temperature range, water, a certain oxygen level.

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Frequently the presence of these factors is not sufficient, and other conditions have to be fulfilled. Given the necessary environmental conditions, growth will be resumed after a certain time, and the radicle, or in some cases the hypocotyl, will start to penetrate through the coverings of the dispersal unit; from then on processes of growth and development will usually continue.

We define as germination processes those which occur from the moment the factors necessary to break dormancy are supplied until the time when growth is resumed (considerable time may elapse before the radicle protrudes through the seed-coat[2–4]). This allows us to differentiate between processes occurring during resumption of growth and continuation of growth, a distinction the usefulness of which has been pointed out repeatedly[5]. Unfortunately, since the most spectacular point in all of these processes is the protrusion of the radicle, there has been a tendency to regard this as the starting point of germination. Many papers dealing with 'changes occurring during dormancy-breaking' actually describe changes occurring during growth.

The study of seeds which germinate only after some special condition is fulfilled throws light on particular steps of the germination process[5]. Let us, therefore, examine some of the different factors which may be necessary for the germination of seeds (for a more complete coverage of this topic, see[1, 6, 7]).

Removal of, or treatment of the surrounding layers will in some cases be effective. The reason for the incapacity of the embryo to develop, even when the three 'primary' requirements —water, oxygen, optimal temperature—are present, may be located not in the embryo itself, but in the surrounding coats, and by removing them completely or partially, or by otherwise treating them, the germination block may be overcome. The ways in which the surrounding layers may influence the embryo are various: the layers may be hard and impermeable to water, as in the seeds of the Leguminosae, thereby preventing germination. Or they may decrease gaseous exchanges as *e.g.* in the cocklebur and many grasses.

In another group, the coats of the dispersal unit exert their influence through substances which inhibit the growth and development of the embryo[3, 8]. These inhibitory substances have actually been found everywhere in the dispersal unit, including the embryo itself, but on this latter point we shall hear in more detail from Dr. Poljakoff. However, in many cases, the embryo germinates readily after excision though it fails to germinate while enclosed in its coats because of inhibitory substances present in the endosperm or other layers. In such a case, even a small piece of endosperm adherent to the embryo may prevent it from growing. The natural substances cited as inhibitory include alkaloids, unsaturated lactones, unsaturated acids, ammonia, KCN and many others. It is, however, not always clear whether the inhibitory substance has a specific activity or whether the resulting inhibition is an osmotic effect. Lerner, Mayer and Evenari[9] have shown that both effects may occur simultaneously, the main effect being either osmotic or specific.

In a large variety of seeds, the inability to germinate cannot be ascribed directly to the influence of the seed coats, and even if the coats may be involved in the chain of events which eventually lead to germination, a treatment has to be given which will affect the embryo itself. This treatment may be prolonged dry storage, at the end of which a seed which did not germinate when imbibed immediately after harvesting will now do so. In order to break the dormancy during this process of after-ripening, different temperatures may be needed at different times[7].

In still other cases, a period of moisture at low temperature may be needed to awaken the embryo, a process which is known as stratification. With other seeds, alternating temperatures may be needed. The similarity between these temperature requirements and those for breaking the diapause in insects are obvious[7].

In many cases, seeds have a strict light requirement which is influenced by temperature (for review on this aspect, see[3, 7, 10]).

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Within certain temperature ranges the seeds have a high germination percentage only in darkness or after exposure to a certain amount of light, both qualitatively and quantitatively definable. In a number of seeds, furthermore, a photoperiodic regime can increase germination.

What makes matters complicated is that frequently, in a given dispersal unit, not one mechanism only is working but many are coupled together, so that very restricted conditions have to pertain to allow the seeds to germinate. To gain an understanding of this aspect the problem is best approached ecologically and we shall hear more about this from Dr. Koller.

Another complication is that although the embryo as a whole is a biological unit, various parts of it may respond differently. A certain treatment may allow growth of one organ, let us say the radicle, but may not be sufficient to allow further development of the hypocotyl. Again, this requires the existence of different and specified conditions at various times.

All this shows that the state of dormancy is a question of equilibrium between various mechanisms and not a case of one mechanism alone being blocked, or of the presence or absence of a certain substance. One should study, therefore, the interaction of various phenomena previous to, and after, dormancy-breakage, in order to get an overall picture of what is going on in a certain organism; the drawback being that a particular organism may be well fitted to give information on one aspect but not on another.

In order to examine details of a single factor, for example, light-mechanism, it has been worthwhile to study this mechanism in different organisms, and not only in regard to germination alone. On the other hand, when nitrogen-metabolism is studied in seeds rich in protein, and carbohydrate-metabolism in those rich in starch, there is a certain danger in transferring conclusions from one type of seeds to the other.

We have concentrated our efforts on a single object, the lettuce seed, where all the aspects are equally difficult to study. Instead of giving examples of differences between states

before and after germination from various plants, let me tell you something of the changes known to us to occur in one single organism, the light-sensitive lettuce seed.

The dispersal unit of the lettuce seed, an achene, is composed of an embryo, the cotelydons of which serve as storage organs, a two-layered endosperm, a seedcoat, and a thick-walled fruit coat which, in case of the light-sensitive variety which will be discussed here, contains a brownish-black pigment[11].

When the seed has been imbibed in water, germination depends both on light and temperature conditions. Below approximately 18°, seeds germinate to a high degree in darkness, and light, therefore, increases the germination percentage only to a small extent.

Between approximately 20–28° the seeds require light for a high percentage of germination and remain dormant in the dark. The light requirement is strongest in freshly harvested seeds, and decreases with age of the seed. A mechanical treatment, such as removing or pricking the coats and the endosperm, or chemical treatment with *e.g.*, thiourea, will cause germination even in the dark.

Above approximately 30°, dormancy cannot easily be broken by light, but germination will result after various other treatments such as stratification for a few days previous to imbibition, high oxygen pressure or, again, removal of the endosperm.

One of the most effective ways of overcoming the thermodormancy of lettuce seeds was found by Thornton[5], who states that lettuce seeds at 35° will germinate normally in an atmosphere of 40–80% $CO_2$.

We can already see here the interaction of various mechanisms[10]. At low temperatures, a photomechanism is taking place indicated by the slightly higher germination of illuminated seeds. At the same time other factors are at work which allow the seed to germinate in darkness. At higher temperatures, these factors cannot any longer break the dormancy, which however can still be overcome by the light mechanism. At still higher temperatures even this mechanism is inhibited. Furthermore, the fact that

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dormancy can be broken by changes in gas pressure or by removal of the endosperm points to this structure as a factor in dormancy. I shall return to the influence of the seed coats on germination of the lettuce seed later.

Part, if not all, of the light mechanism is connected with the well known reversible Red–Far Red reaction. The light effective in bringing about germination is red light of about 6500–6800 Å, the promoting effect of which can be completely overcome by illumination by Far Red light, of wave length 7200–7500 Å, and vice versa[3, 7, 12]. It is the same reaction that is active not only in the control of germination of a large number of seeds, but also in a wide array of other phenomena and can be regarded 'as a general factor of growth control'[7], thereby relating seed germination to other biological phenomena.

In this reaction, a single relatively short illumination is necessary to bring about the promoting or inhibitory effect. The sensitivity to this illumination depends on a number of factors, among others the time lapse between imbibition in darkness and illumination. At a temperature of 26°, lettuce seeds start to respond to light as little as approximately 10 min after imbibition and the sensitivity rises for approximately 8 h, and then decreases[13]. This time curve is not typical for all light-sensitive seeds. For *Amaranthus* seeds it was shown that several days are needed before sensitivity reaches its peak, but in this case the sensitivity is maintained even after two months[14].

As regards illumination with white light, a distinction should probably be made between the effects of a single short illumination and those of prolonged continuous illumination[10]. For example, Kadman-Zahavi[15] found in *Amaranthus retroflexus*, at a certain light intensity, 32% germination in the dark, 3% in continuous light and 92% after a single short illumination. In general, the main difference seems to be that in the case of short illuminations with white light, germination rises with light intensity, whereas with continuous illumination, germination response is inversely proportional to it[10, 15]. Attempts have been made to explain this, assuming that the Far Red radiation

present in 'white' light may be responsible for this, since it has been found that short and prolonged Far Red irradiations differ in their effect. In the *Amaranthus* seeds, a short illumination with Far Red is reversible by a subsquent red irradiation, whereas the inhibition by Far Red given over a prolonged period is not reversed by an immediately following illumination with red light[15]. Therefore, when white light is given over a prolonged period, the 'prolonged Far Red' effect may be dominant over the stimulatory red effect and cause the inhibition.

The effect of the prolonged Far Red irradiation, however, decreases with time: as mentioned, red light given immediately after the prolonged Far Red will be without effect; but when the seeds are kept for some time in darkness after the Far Red illumination and only then exposed to red-light, germination will occur.

A somewhat similar difference between short and prolonged Far Red irradiation has been found also in certain lettuce seeds. The seeds of the 'Progress' variety are light sensitive only when very young. Older seeds have no light requirement and when imbibed, will germinate readily both in the dark and in the light. Red light has no effect on those seeds, because of the already high 'dark germination', but also a short Far Red illumination is without effect and will not cause inhibition of germination. However, when the seeds are exposed to prolonged Far Red irradiation, their germination is almost completely inhibited. This inhibition is reversible by red irradiation but much stronger doses of red light are required than in the case of the usual 'Red–Far Red reaction' (Table I). Both the facts, that in this case (1) a short Far Red irradiation is without effect, whereas prolonged Far Red irradiation inhibits, and (2) that Red in much stronger doses than usual is needed to overcome the Far Red effect, may be taken as an additional indication that the 'prolonged Far Red' effect is different from the effect of Far Red in the well known short Red–Far Red reaction[10]. Still, it has yet to be shown definitely whether this difference exists in reality or whether the mechanism of the short Red–Far

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TABLE I

GERMINATION PERCENTAGES OF LETTUCE SEED 'PROGRESS'

illuminated with Far Red for 2($FR_2$), 5($FR_5$) and 7 days ($FR_7$) and then transferred to darkness (D) or illuminated with red (250 ft.-c.) for 5($R_5$), 20($R_{20}$) or 40 seconds ($R_{40}$) (Evenari[10]).

| | $FR_2$ | $FR_5$ | $FR_7$ |
|---|---|---|---|
| D | 3 | 4 | 6 |
| $R_5$ | 10 | 9 | 6 |
| $R_{20}$ | 81 | 27 | 19 |
| $R_{40}$ | 80 | 31 | 26 |

The germination of the untreated seeds in darkness varies between 90–93% (ref.[10]).

Red reaction may not, after all, be responsible also for the above mentioned phenomena. The case of the Progress seeds is interesting also from another point of view. When these seeds are imbibed in a solution of coumarin, they behave exactly like the light-sensitive Grand Rapids variety[16]. Thus, in cases in which one does not normally find light sensitivity, under certain conditions light may still have a profound influence.

What makes understanding of the light mechanism still more difficult is that blue light too, in many cases, has a distinct influence, the nature of which is yet unclear.

Without any doubt, however, the most important light mechanism is the reversible 'short' Red–Far Red reaction. Until very recently, attempts at identification of the pigment responsible for this reaction were unsuccessful. A few months ago, the photometrical detection of this pigment in living tissue of maize shoots was reported by Butler, Morris, Siegelman and Hendricks[17]. The same group also succeeded in separating the pigment from the tissue by methods of protein chemistry. A spectral shift could be observed after both Red and Far Red irradiation. This is a most important advance since it enables us

to tackle the nature of the enzymatic action involving this pigment through which germination and growth process in general are controlled.

Among the many data available on light mechanism in the lettuce seed, I shall mention only two: First, that light exerts an influence not only on the imbibed, but also on the 'dry' seed[18]. This influence, which is strongly dependent on the relative humidity of the air, is probably related to the internal water content of the seeds. Seeds stored at a relative humidity of 60–80% in the light and subsequently germinated in the dark gave a higher germination percentage than those stored at the same relative humidity in the dark. No such effect of light could be found in seeds stored at 20–30% relative humidity. This indicates that the relatively low water content of the dormant seed has to be raised only slightly in order to make the seed respond to external influences.

Secondly, the reversibility of the Red–Far Red reaction in the lettuce seed depends on the time interval between the two illuminations. After 10 min of darkness, Far Red cannot any longer reverse the effect of red[10]. This shows that only the first steps in dormancy breaking are influenced by light. Once the reaction chain has started, light, at least in lettuce, does not have any influence on further events.

Considering the changes induced by these light mechanisms which finally cause resumption of growth, one of the most important factors is, of course, water uptake. It might be imagined that dormant and non-dormant seeds would take up different amounts of water when imbibed, *i.e.* their water uptake would differ in light and darkness. This, however, is not the case, and water uptake under both these conditions proceeds almost indistinguishably until growth starts in the light-treated seeds, after approximately 14 h; *i.e.*, the light treatment does not change water uptake in the seeds.

Since a good deal of work has been done on chemical inhibitory and promotory agents for germination, let me say a few words on the effect of some of these substances in lettuce seed

*References p. 190*

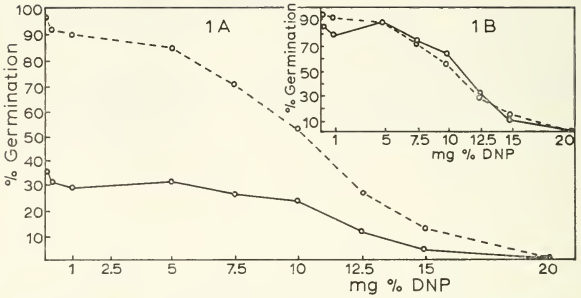


Fig. 1. Germination percentage of lettuce seeds (Grand Rapids) in different concentrations of dinitrophenol. 1 A: absolute germination percentages; 1 B: germination percentages related to the germination percentage in water as 100. (– – – –) after illumination; (——) in darkness.

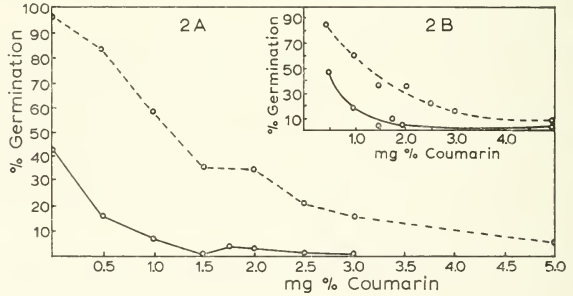


Fig. 2. Germination of lettuce seeds in different concentrations of coumarin. Explanation as in Fig. 1.

germination. Comparing the effect of coumarin with that of dinitrophenol, both of which are germination inhibitors, Figs. 1 and 2 show the amount of seeds germinating as a function of various concentrations after a light treatment and in darkness. Since the percentage of germination of light-treated seeds is always higher than of seeds kept in darkness, this seems to indicate that light can to some extent overcome the effects of

both the inhibitors. However, if we take the germination of the controls (in water) as 100, and express germination in the presence of the inhibitors as percentages of the water control, we see that the inhibitors differ in their action. Higher concentrations of dinitrophenol depress germination equally in darkness and after light treatment, while coumarin at all concentrations inhibits germination much more strongly in the dark, showing that light can to a certain extent obliterate the effect of coumarin. Since, as was mentioned earlier, the light mechanism influences only the early steps in the chain of reactions leading to germination, it would follow that coumarin acts on these early steps, whereas dinitrophenol affects processes which are not any longer under the control of the light mechanism. 2–4 D, according to Evenari, behaves in a similar way to coumarin[10].

Since the question of respiration occupies so eminent a place in the symposium, let us say something about respiration in dry, resting seeds and in germinating ones. Do resting seeds respire or not? There seems to be no doubt that the level of respiration in 'dry' seeds is largely a matter of moisture content. In general, down to a water content of approximately 10%, there always seems to be a small but measurable $CO_2$ output and $O_2$ uptake. When moisture is reduced below this level, the gas exchange is dramatically slowed down, and very frequently cannot be measured, although the seeds remain viable for a long time. As has been pointed out frequently, this only means that no measurable amount of $CO_2$ can be detected and not that gas exchange actually ceases.

James[19] cites in this regard a comment of F. F. Blackman: 'It may well be that there will not be enough $CO_2$ produced to be detectable in ten years, but who shall say that change has ceased? Our methods of analysis which demand a large aggregate of molecules for any demonstration are incapable of settling this philosophical question'. Even in those cases where small traces of $CO_2$ output can be measured, two important questions arise. First, if this $CO_2$ output is indicative of what usually is defined as respiration and secondly, how far it may not be due

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to micro-organisms. James cites Blackman as having considered that the $CO_2$ output may be due to a purely photochemical reaction, in which the organisation necessary to maintain viability is destroyed, since viability is lost without any considerable depletion of seed reserves. James concludes: 'So far as dormant seeds are concerned we might therefore still be up the horns of the old dilemma: either they live without respiration, with extreme sluggishness no doubt, but still live, or, as Claude Bernard thought, they cease to live and come alive again'.

However, when we consider the respiration of wet seeds, we are on firmer ground, since water uptake is always accompanied by an increase in respiration. During the first hour of imbibition there is a sharp rise in respiration, frequently with large and abrupt changes of RQ, giving quite extreme values. It has been shown, however, that these changes are due more to the physical conditions under which the seeds are imbibed than to changes in the respiratory apparatus. Later on, a more or less steady trend is reached, until the time when growth processes are resumed. In cases where no renewal of growth occurs, respiration rate goes down with a decrease of viability in the seeds[19].

Is there any difference between the respiration rate of imbibed dormant and non-dormant seeds? Let us again consider lettuce seeds for illumination of this problem. Both dormant and non-dormant seeds can be compared under exactly the same conditions. The seeds are imbibed in darkness in Warburg flasks, a dose of light is given after two hours to certain of the flasks and readings are made. Since the first single mitotic division occurs in the germinating seeds after approximately 12–14 h, only a short time before the radicle protrudes through the seed coat, there is ample time to look for differences between dormant and non-dormant seeds during time of germination.

The curves in Fig. 3 show hourly rates of $CO_2$ uptake and of $CO_2$ output in seeds which were briefly illuminated 2 h after imbibition. These rates are expressed as percentages of the gas exchanges in these seeds which were kept all the time in the

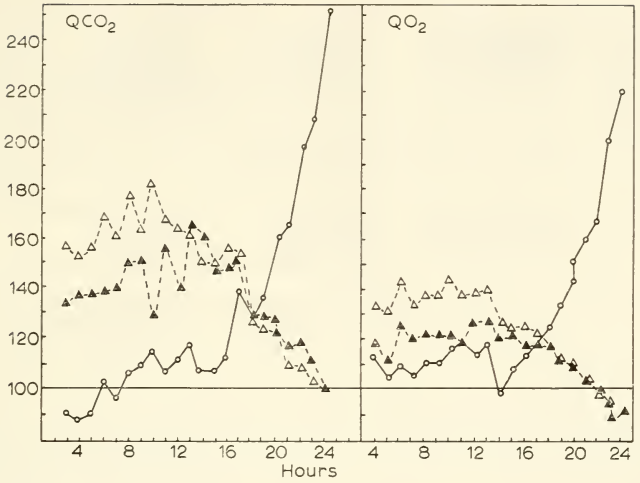


Fig. 3. $QO_2$ and $QCO_2$ of lettuce seeds (Grand Rapids). The straight line at 100 is the $QO_2$ and $QCO_2$ respectively in darkness and water, to which all the other values are related. $QO_2$ and $QCO_2$ respectively of seeds imbibed in water, which were illuminated for 30 seconds with 250 ft.c. of red light after 2 h of imbition (○—○). $QO_2$ and $QCO_2$ respectively of seeds imbibed in 10 mg% dinitrophenol and illuminated (△—△). $QO_2$ and $QCO_2$ respectively of seeds imbibed in 10 mg% dinitrophenol in the dark (▲—▲).

dark. In illuminated seeds, imbibed in water, there is a small rise in rate during the first 12 h; this was found to be highly significant. The total amount of gases taken up or evolved during this 12-h period is higher, but not significantly so, in illuminated than in 'dark' seeds. This is because in discrete batches of illuminated seeds, the rate of gas exchange sometimes starts at a lower level and sometimes at a higher one than in 'dark' seeds. The main consideration is that respiration is affected in illuminated seeds. The difference in respiration between illuminated and non-illuminated seeds is seen in the tendency to rise of the exchange rate and not necessarily in the overall amount of gas exchange. It can, therefore, easily be overlooked, especially if

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only respiration at specific times is considered in different batches of seeds.

This difference in respiration behaviour between 'light-treated' and 'dark' seeds may also be found in the presence of inhibitors. It is evident from the Figure that in the light-treated seeds, a 10 mg % dinitrophenol treatment results in a much stronger gas exchange than with 'dark' seeds, although germination is inhibited in both cases.

In the presence of coumarin, light has a similar tendency to increase respiration, and the tendency increases with the degree of inhibition of germination produced by the inhibitor. In general, light without doubt affects the respiratory apparatus during the germination period.

It was mentioned earlier that seeds with their coats removed or pricked will germinate even in darkness. What about respiration in these? It turns out that their respiration during the first hours is much higher than in whole seeds. Thus, we again arrive at the possibility that gas exchange is limited by the coats and that all that light does is to render the endosperm more penetrable to gases. Doubts have been expressed whether it is the actual removal or pricking of the endosperm which brings about germination, or whether the stimulation may not be due to the squeezing of the embryo itself during the process of coat removal[7]. Using deuteron irradiation with a controlled penetration depth into the seed, it was possible to show that it is both necessary and sufficient to affect the endosperm alone in order to bring about germination[20, 21] (Fig. 4). Incidentally these experiments also showed that there is not necessarily a correlation between germination process and subsequent growth. A small dose of deep-penetrating deuterons bombarding the embryo itself leaves the endosperm unaffected, and therefore does not change the germination pattern, but causes a decrease in subsequent growth. On the other hand, a strong dose of deuterons applied to the endosperm alone will allow complete germination in darkness, without affecting the growth of the seedlings. This deuteron-induced germination cannot be reversed

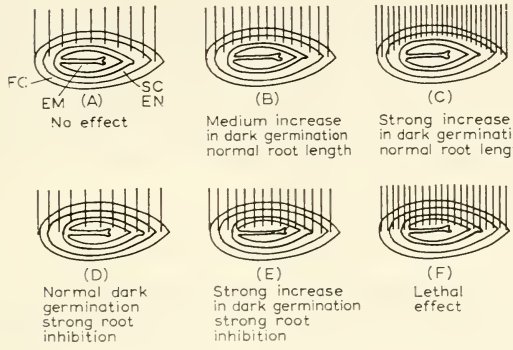


Fig. 4. The effects of deuteron irradiation on lettuce seed germination and subsequent root growth when applied to different depth levels. A to C: deuterons penetrating into endosperm only. D to F: deuterons penetrating deep into embryo. A and D: Weak dose; B and E: medium dose; C and F: strong dose. FC: Fruit coat; SC + EN: seed coat and endosperm; EM: embryo. The figures are not drawn to scale[20].

by Far Red irradiation, which means that a different mechanism is involved.

This, however, does not mean that all the treatments we have mentioned affect the endosperm itself. For example, Koller[22] has shown that in certain plants the radicle itself is the light receptor, and recently good evidence in this respect has been brought forward for lettuce seeds by Ikuma and Thiman[23].

Most probably, therefore, the chain of events starts in the embryo, possibly in the same cells which later on will be the first to grow and divide, but leads in the end to an effect on the endosperm, thereby resulting in increased gas exchange and perhaps reduced mechanical pressure on the embryo. As to what goes on in between these stages, our knowledge is less than scanty. It involves enzymatic activities which will be the subject of another lecture. Of course, I would be leaving this picture still more incomplete, were I not to mention that resumption of growth after a dormant stage certainly also involves changes in

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a very delicately balanced equilibrium between growth substances and their specific inhibitors. This too will be discussed separately. Incomplete as this résumé is, I hope it still shows that dormancy in seeds cannot possibly be dependent on one or a few substances only, or on one mechanism only, but that it is a state dependent on a large number of systems, which we are still unable to comprehend fully.

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# BIOCHEMICAL CHANGES IN BREAKING AND INDUCING DORMANCY IN SEEDS

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The term dormancy is one which is used in various senses by various authors and this is particularly the case as regards the dormancy of seeds. In this paper, it is intended to use the term as referring to a population of seeds, specifically here lettuce seeds. If such seeds are placed under specified conditions of temperature and moisture, usually 26° in the dark, on moist filter paper, a certain number of them will germinate. The percentage of seeds which germinate under these conditions gives a measure of the dormancy of this seed population. This percentage can be altered in various ways, *e.g.*, it can be increased or decreased by a number of physical and chemical agencies. Those which increase the percentage of germination may be considered as dormancy-breaking, while those which cause a decrease without, however, affecting irreversibly the eventual ability of the seeds to germinate when some other treatment is given, will be considered as inducing dormancy. From this it is immediately clear that, in referring to biochemical changes occurring during induction and breaking of dormancy, what is in fact being considered is the overall biochemical behaviour of a population of seeds under different treatments. In fact, the action of a dormancy-breaking and dormancy-inducing substance is always considered relative to the behavior of the untreated seeds. In no case is there any certainty that a specific change has occurred within any one given seed, and indeed, no attempt has ever been made to try and analyse individual seeds. This is for two reasons.

First, lettuce seeds are extremely small and inconvenient to handle individually. The second and more important reason is that there is no way of foretelling whether any given seed will or

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will not germinate without awaiting some indicative external sign, usually the protrusion of either the radicle or plumule from the seed coat, and by the time this has occurred germination has been completed. No other clear indication of whether germination will or will not occur has been found for any seeds. Biochemical tests or histochemical staining have not proved adequate to answer this question.

The two treatments of the seeds which we will now consider are using thiourea, which breaks dormancy, and coumarin, which is a germination inhibitor and in fact induces dormancy. The results to be considered, primarily those of Dr. Poljakoff-Mayber and myself, are concerned with the biochemical changes which occur in lettuce seeds germinated in solutions of these two substances, as compared with those of seeds germinated in water. It may be said at the outset that in no case is there any certainty that these changes are in fact directly related to the dormancy-inducing or -breaking action of the chemicals concerned; in some cases, however, they are suggestive of this.

During germination of seeds there is a general activation of all the enzyme systems in the seeds, due to water uptake. This activation is accompanied by a breakdown of certain storage products within the seeds, and by a general increase in the oxygen uptake. However, not all enzyme systems increase their activity at an equal rate and a few of them in fact rapidly decrease in activity as germination proceeds. Studies of the biochemical changes occurring in the germinating seeds have not yet been extended to all the metabolic processes that occur. Certain vital systems have not as yet been studied at all, whereas others have been shown to be of only secondary importance. It is therefore intended to confine the discussion to the breakdown of certain storage materials and to the possible oxidative pathway occurring during germination.

It became clear fairly early in the investigation that in lettuce seeds the first substrate to be used during germination is the small amount of sucrose present[1]. The breakdown of this sucrose is not inhibited by either coumarin or thiourea during

the early stages of germination. However, while in germinating seeds glucose appears as the sucrose disappears, this accumulation of glucose is completely blocked in seeds whose germination is prevented by coumarin (Table I).

TABLE I

THE EFFECT OF THIOUREA ON THE METABOLISM OF LETTUCE SEEDS AND SEEDLINGS

Seeds germinated in thiourea (1250 p.p.m.) at 26° in the dark

| *Metabolite* | *Activity effect of thiourea* | *Reference* |
|---|---|---|
| Sucrose utilisation | Practically no effect | 1 |
| Fat utilisation | Slight inhibition | 2,8 |
| Glucose accumulation | Practically no effect | 1 |
| Free fatty acid liberation | Stimulation in later stages of growth | 2 |
| Volatile fatty acids | Slight stimulation | 2 |
| Phytin breakdown | No effect | 6 |

The principal storage material in lettuce seeds are lipids. The major decomposition of the lipids occurs at a fairly late stage of germination, but small amounts are probably already broken down at earlier stages. Both coumarin and thiourea cause rather extensive changes in the metabolism of the fats. Coumarin in general prevents the breakdown of lipids in the seeds[2, 8] and at the same time the appearance of free fatty acids is also delayed or prevented while volatile ones increase. These latter need not necessarily and probably in fact do not arise from the lipids directly.

The enzyme systems which are known to be active in the seeds in lipid breakdown are two lipases, one having maximal activity at acid pH and the other maximal activity at neutral pH. Neither of these enzymes show an increase in activity in seeds treated with coumarin (Table II). In contrast, thiourea inhibits the acid lipase, which in normal germination begins to increase in activity only at a fairly late stage of the germination process, while the neutral lipase activity rises initially and then

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TABLE II

THE EFFECT OF COUMARIN *in vivo* AND *in vitro* ON THE ENZYMATIC ACTIVITY OF VARIOUS HYDROLYTIC ENZYMES IN GERMINATING LETTUCE SEEDS

*In vivo:* seeds germinated in coumarin solutions (100 p.p.m.). The enzyme assays were carried out in the absence of coumarin.
*In vitro:* seeds germinated in water and coumarin was added to the reaction mixture.

| *Enzyme* | *Effect on activity in vivo* | *Effect on activity in vitro* | *Reference* |
|---|---|---|---|
| Lipase, neutral | Inhibits increase in activity<br>Activity remains as in dry seeds | Inhibits strongly | 3 |
| Lipase, acid | Inhibits increase in activity<br>Activity remains as in dry seeds | No effect | 3 |
| Phytase | Strong inhibition | Slight inhibition | 6 |

falls again in seeds germinated in thiourea[3] (Table II, III). Despite these changes in the lipase itself the fat metabolism is far less profoundly affected by thiourea. Total breakdown is

TABLE III

THE EFFECT OF THIOUREA *in vivo* AND *in vitro* ON THE ACTIVITY OF SOME HYDROLYTIC ENZYMES IN GERMINATING LETTUCE SEEDS

*In vivo:* seeds germinated in thiourea (1250 p.p.m.). No thiourea present in the reaction mixture.
*In vitro:* seeds germinated in water and thiourea added to the reaction mixture.

| *Enzyme* | *Effect on activity in vivo* | *Effect on activity in vitro* | *Reference* |
|---|---|---|---|
| Lipase, neutral | Increase, then decrease | — | 3 |
| Lipase, acid | Inhibition progressing with time | Inhibition | 3 |
| Phytase | No effect | No effect | 6 |

hardly affected, free fatty acid formation is slightly depressed and volatile fatty acid formation is somewhat depressed. In this case, therefore, dormancy breaking with thiourea and dormancy induction with coumarin have opposite effects only on one part of the system, that is, the free volatile fatty acid. Although this fatty acid was not identified with certainty it seems probable that it is either acetic or lactic acid. In either case this would make it probable that its origin is closely connected with respiratory mechanisms. It seems more than doubtful in any case, that the changes observed are directly related to dormancy breaking or induction.

Seeds which germinate in the dark in water show an increasing oxygen uptake which starts almost immediately after the seeds are placed in the water. A number of respiratory enzymes are active even before the seeds are imbibed. These enzyme systems can be shown to exist if the dry seeds are extracted with water: the possibility that they become activated during extraction with buffer cannot be excluded. Those systems which have been shown to be active in the dry seeds, keeping the above limitations in mind, are a cytochrome oxidase, a number of dehydrogenases, a DPNH oxidase, catalase, peroxidase and a phenolase system, as well as what is possibly an ascorbic acid oxidase[4, 6]. Some of these enzymes increase greatly in activity as germination proceeds while others, particularly the glucose phosphate dehydrogenase[10], the phenolase and the DPNH oxidase[15], do not, but even show a decrease. In this respect it is worthwhile to recall that during germination the number of cells in the seed or embryo increases. Therefore, the failure of an enzyme system to increase as germination proceeds implies that the average amount of enzyme per cell is decreasing. It is of course also quite possible that in some parts of the embryo the system does not develop at all while in others it maintains its original activity.

It has been shown that during normal germination, the tricarboxylic acid cycle activity of mitochondria prepared from the seeds is very low initially, and then increases as germination

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TABLE IV

THE EFFECT OF THIOUREA *in vivo* AND *in vitro* ON THE ACTIVITY OF OXIDATIVE ENZYMES IN GERMINATING LETTUCE SEED

*In vivo:* seeds germinated in thiourea (1250 p.p.m.). No thiourea present in the reaction mixture.

*In vitro:* seeds germinated in water and thiourea was added to the reaction mixture.

| *Enzyme* | *Effect on activity in vivo* | *Effect on activity in vitro* | *Reference* |
|---|---|---|---|
| Ascorbic acid oxidase | Inhibition | Inhibition | 6 |
| Phenolase and coupled oxidations | Slight stimulation or inhibition depending on substrate | Strong inhibition or no effect depending on substrate | 4 |
| Catalase | Inhibition | No effect | 1 |
| Peroxidase | Strong stimulation | No effect | 9 |
| Dehydrogenases | Very slight inhibition | Practically no effect | 5 |
| DPNH oxidase | Inhibition | Inhibition | 15 |
| Lettuce mitochondria | Stimulates the development of the citric acid cycle in very young seedlings but has no effect on the activity of mitochondria isolated from other seedlings | No effect | 13 |

proceeds[5, 12, 13] (Table IV). This finding must be contrasted with what occurs in seeds whose dormancy is altered.

Coumarin, generally speaking, retards or prevents the development of, or increase in activity of the above enzyme system. In no case has a direct effect of coumarin in any of the systems mentioned *in vitro* been found. This observation can be extended by noting that the entire glycolytic system functions

normally in peas germinated in coumarin[7, 11]. Thus it appears that coumarin does not act by directly suppressing some specific enzyme. There are, however, indications that it may act indirectly through its effect on release of inorganic phosphorus from phytin[6] and also on ATP metabolism[12] and may thus in some way control respiration, or rather the energy supply to the seeds, by interfering with the rate-determining or controlling steps during oxidation of substrates.

The changes in the oxidative system brought about by treatment of the seeds with thiourea are far more widespread. Poljakoff-Mayber and Evenari[13] showed that the rate of oxidation of tricarboxylic acid cycle intermediaries was much more rapid by mitochondria isolated from seeds germinated in water. Thus it seems that the tricarboxylic acid cycle enzymes become active much more quickly in the presence of thiourea. Preliminary results indicate similar effects for the cytochrome system, or at any rate for cytochrome oxidase. In contrast to these systems which become more rapidly active, a number of systems are inhibited. Oxidation of ascorbic acid and of DPNH by extracts of lettuce seeds is completely prevented when the seeds are germinated in thiourea. The systems responsible for this oxidation are situated in the soluble part of the cell[11, 15].

The oxidation of phenolic substrates is altered by germination in thiourea (Table II). Extracts of seeds germinated in thiourea do not show any blackening even on prolonged standing. These extracts still contain an active phenolase which is not itself inhibited by thiourea[11]. However, the oxidation of quinol, apparently by a coupled oxidation, is completely inhibited both by *in vivo* and *in vitro* treatment.

It is possible that such coupled oxidations and also ascorbic acid oxidase and DPNH oxidase, normally mediate part of the electron transport in the seeds. There is at present little evidence to show that in such electron transport systems oxidative phosphorylation occurs. The disruption of such a system by thiourea, leading to the more rapid entry into operation of the normal Krebs cycle and cytochrome system as energy-providing

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mechanisms, may therefore be possible. One action of thiourea in breaking dormancy may then be the following: Normally, in the initial stages of germination, the seeds are basing most of their oxidative processes on the oxidation of glucose-6-phosphate, probably via the pentose shunt and their electron transport system is based on transport via ascorbic acid or phenols, with either a coupled oxidation with phenolase as the oxygen carrier, or by direct participation of ascorbic acid oxidase and DPNH oxidase. At a later stage of the germination process these systems are replaced by the tricarboxylic acid cycle, glycolysis and the cytochrome system for electron transport. If the seeds are treated with thiourea, this whole set of changes is brought into operation with much greater rapidity, because some of the other systems are depressed, and thus, it is tempting to suggest that thiourea breaks dormancy by indirectly calling into operation more rapidly the energy-releasing processes in the seeds.

To put forward this hypothesis at the present stage is somewhat premature, as the evidence is far from conclusive: it is, however, a possible working hypothesis which can be tested experimentally. It is by no means suggested that these are the only changes which are induced by thiourea. That other changes occur is becoming clear today, and it is possible that in order to break dormancy, a number of biochemical events have to occur simultaneously. There is some evidence that the hormonal system is also altered by thiourea treatment.

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*Note added in proof:* Recent work has shown that ascorbic acid oxidase is absent from lettuce seed and ascorbic acid is oxidized by phenolase in a coupled oxidation (Stavy and Mayer, *Bull. Research Council Israel*, in press, 1961.

## DISCUSSION

Halvorson Jr.: Your finding of a shift to the tricarboxylic acid cycle during germination is very interesting, as the opposite is the case with bacterial spores. Is the system at this point sensitive to antimycin A, particularly in the presence of thiourea? Is germination stimulated by Krebs cycle intermediates?

Mayer: I regret to say that we have not tried the sensitivity of antimycin A. As to your second question, there is no clear stimulation of germination by Krebs cycle intermediates.

Avi-Dor: I wanted to ask you about the increase of activity of the tricarboxylic acid cycle in the mitochondria, noted by you during germination. This increase might be caused by an increase in either the number or the size of the mitochondria, and not by an increase in enzymatic activity.

Poljakoff-Mayber: We hope to be able to solve this problem with the help of electron microscopy, by observing both the number and the size of mitochondria in different stages of germination. In dried seeds the oxygen uptake per mg nitrogen is very low. It increases during the process of germination from 2–5 $\mu$l/min/mg nitrogen to several hundreds of $\mu$l/min/mg nitrogen.

Kohn: I do not understand why light should have any effect on seeds, since they have to be in the ground before they germinate. Could it not be the effect of infrared or heat radia-

tion? My second question: is a case known in plants, similar to that in insects, where the life history of one stage of development may affect the dormancy of the next stage?

KLEIN: Some seeds will only germinate on the surface of the soil and need light for it. It is, however, not a heat effect, as a short illumination with weak red light will give this effect. The effect of white light preventing germination might be protective for seeds germinating underground. As to your second question, there are cases where the photoperiodic regime of the mother plant has an influence on the seeds.

KOLLER: A case of the photoperiodic regime of the mother plant, affecting the dormancy of the seed has been reported by Lona on *Amaranthus;* seeds formed on long days were more dormant than seeds formed on shorter days.

HESTRIN: I would like to point out that botanists working in morphology describe plant structures in their minutest detail. Once, however, they deal with biochemistry, they are satisfied with effects caused by whole organisms. For example, increases and decreases in activity were noted and ascribed to mitochondria in lettuce seeds, without any indication as to the origin of these mitochondria, the work being performed on a mixture of tissues. It seems to me that this biological work can be brought to a biochemical level by the use of single cells or of homogeneous tissue. Would unicellular algae or pollen grains be suitable in the study of the dormant state?

I would also like to propose a hypothesis as to the mechanism responsible for induction and breaking of dormancy: suppose the cell contains in one compartment nutrients and in another enzymes. The breaking of dormancy would be the rupture of the separating wall, the induction of dormancy the erection of such a separating wall.

POLJAKOFF-MAYBER: The lettuce seed we have been working on consists of the embryo and tissues which are to a large extent degenerated. It follows that the mitochondria must have come from the embryo. Within the embryo the concentration of mitochondria may vary, but such observations would require

larger seeds. I am doubtful whether pollen grain would be of help in this study, since they are too simple compared with seeds. We would miss all the elements of interrelations.

MAYER: I would like to defend myself against some of the attacks made by Prof. Hestrin. I pointed out that we worked with mashed up tissues, and that this fact limited the value of our results. However, in this way we obtained indications which will enable us to determine the specific parts of the tissue involved.

HALVORSON: Is there any difference in the heat resistance of the seed before and after germination?

KLEIN: I am afraid this has not been tested.

GOLDWASSER: I would like to suggest the use of the fluorescent antibody technique to detect the site of enzyme formation, provided you can prepare these enzymes in a reasonably pure form.

KOLLER: In regard to Prof. Hestrin's proposal for work on homogeneous tissues, I would like to suggest fern spores. They might be preferable to pollen grains, since they are activated by light in the Red–Far Red system.

NACHMONY: I would like to suggest the use of *Bryophyta* as suitable organisms for the biochemical study of these problems. In these plants dormancy can be induced and broken by photoperiodic treatment. The fact that almost every stage of the life cycle, including young sporelings, is able to react to the same treatment, may indicate that this ability is common to almost all cells, thus making the whole organism nearly homogeneous in this respect.

# REST IN BUDS OF WOODY PLANTS*

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Continuing our previous discussion, it seems desirable to define 'dormancy'. Dormancy in plants is a suspension of visible growth accompanied by a slowing down of the rate of metabolism. We should distinguish between two types of dormancy. One, quiescence, is caused by unfavourable external factors; when these factors are removed growth is resumed. The second type of rest may be defined as a suspension of growth due to internal factors. Even if the plant is provided with favourable conditions it will not resume growth, unless some internal factor is changed or some block removed.

Those of you who came here from Jerusalem enjoyed, no doubt, the profuse bloom of plum trees in the hills, while you may have noticed that the trees were still dormant in the coastal plain. This is typical of Israel, where after the usual warm winter, bloom in colder areas (*i.e.* at higher elevation and at greater distance from the coast) occurs earlier than in the coastal plain. Thus we see in Table I that the bloom of Santa Rosa plums at Rehovot near the sea shore was later by 10 days than the bloom at Jerusalem up in the mountains. These observations are not in agreement with general phenological concepts, according to which bloom occurs earlier in warmer spring weather. The general phenological rule holds true for Israel after exceptionally cold winters, as seen in the Table. Not only is the relative trend as regards time of blossoming in warm and cool areas reversed as compared with that after a warm winter (except for Rehovot), but the average date of bloom is advanced by almost one month.

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TABLE I

EFFECT OF CLIMATE ON DATE OF FULL BLOOM OF SANTA ROSA PLUMS

| *Location* | | *Date of full bloom* | |
|---|---|---|---|
| | | *warm winter* | *cold winter* |
| Jerusalem | (mountain crest) | March 31 | March 10 |
| Affulah | (central valley) | April 3 | March 8 |
| Mishmar H. | (northern coastal valley) | April 5 | March 2 |
| Rehovot | (southern coast) | April 10 | March 9 |

Thus, a cold winter, a longer period of chilling, reverses the abnormal situation brought about by the warm winter, *i.e.* a certain period of chilling is required in order to terminate rest.

I should like now to show you the experimental effect of chilling. Pelee[1], working in our laboratory, placed resting Kelsey plum shoots for various periods into a refrigerator at 4°. As Table II shows, increased periods of chilling progressively

TABLE II

EFFECT OF DIFFERENT PERIODS OF CHILLING DURING REST ON THE AWAKENING OF KELSEY PLUM BUDS

| *Chilling period (days)* | *Blossoming date* | *Open buds (% of total buds)* |
|---|---|---|
| 0 | April 13 | 7 |
| 7 | April 7 | 15 |
| 21 | March 24 | 17 |
| 56 | March 10 | 29 |

advanced the awakening of buds in spring. One week of chilling put forward bud opening by about a week, while chilling for 56 days advanced wakening by as much as 33 days. Futhermore, chilling not only advanced the date of bud opening, but also increased the number of buds which opened. Seven per cent of buds opened on the control shoots; one week of chilling doubled

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the number, and about four times this number of buds sprouted after the maximum chilling period.

In warm countries, insufficiency of chilling prolongs rest, delaying bloom and fruit maturation, and reducing the number of buds which open. Thus, both the photosynthetic area and the number of blossoms are affected. These phenomena, together with certain additional physiological disturbances caused by prolonged rest, constitute a serious economic factor with the result that certain crops cannot be grown economically in regions with warm winters.

Horticulturists—as in our department—try to solve this problem by methods such as selecting and breeding varieties with low chilling requirements. The artificial breaking of rest represents an additional possibility. Johannsen[2] was the first to do so by means of anaesthesia. Molisch[3] interrupted the rest period by immersing plants in a warm water bath. The physiological mechanism of this treatment was explained by Boresch[4] on the basis of anaerobiosis. For trees, we studied a number of sprays, the effects of which are described in Table III. On the

TABLE III

THE EFFECT OF MINERAL OILS AND CERTAIN DINITRO-COMPOUNDS ON THE AWAKENING OF DELICIOUS APPLE BUDS

| *Spray material* | | *Open buds (%)* |
|---|---|---|
| Unsprayed control | | 12.5 |
| Mineral oil, med. heavy, 4%, | U.M.R.* 100 | 17.7 |
| | 75 | 19.5 |
| | 65 | 30.5 |
| Dinitrocompounds** in med. oil U.M.R.* 75 | | |
| 2,4-dinitro-*α*-naphtol | | 29.7 |
| 3,5-dinitro-*o*-cresol | | 28.7 |
| 2,4-dinitro-6-cyclohexylphenol | | 25.2 |

* U.M.R. = unsulphonatable residue = % saturated constituents.
** Dinitrocompounds = concentrations equimolecular to dinitrocresol, 1.5 % in the mineral oil.

untreated shoots of apple trees only somewhat over 12% of the buds opened. With a mineral oil spray, even when it was completely saturated, we obtained an increase in sprouting of about 50%, although we could not expect from a saturated oil any chemical action. It would seem probable that the inert oil film interferes with the oxygen supply of the cells, again a situation leading towards anaerobiosis. We also investigated the effect of mineral oils with different degrees of unsaturation. Our results show a considerable increase of the rest-breaking action with an increasing proportion of the unsaturated compounds in the oil (lower U.M.R.). Furthermore, we dissolved dinitrocompounds in the mineral oil. All three which we tried—and dinitrophenol which we studied in another series of tests—increased the rest-breaking action of the spray, thus doubling the number of growing buds of the control. Today this dinitrocresol–mineral oil spray[5] is used widely by growers in Israel and in certain other subtropical countries.

The physiological action of dinitrophenol has been reviewed by Simon[6]. Its uncoupling action is caused by inactivation of enzymes concerned with oxidative phosphorylation. Since so fundamental a process may affect cell metabolism at several points, we cannot point to a definite reaction bringing about the breaking of rest. But we do find a common denominator with previously mentioned methods of rest breaking: dinitrocompounds lead cell respiration into fermentative pathways. Bahgat[7] in the laboratory of Bennett broke the rest of pear shoots by holding them under nitrogen and found subsequently in their tissues both ethyl alcohol and acetaldehyde. When he treated dormant pear shoots with alcohol or acetaldehyde, he was able to break their rest. Thus, we see that products of anaerobic respiration are effective in terminating the rest period.

The entry of buds into the resting stage is affected by the length of the daylight period, as originally shown by Garner and Allard[8] and investigated further in the laboratories of Borthwick[9], Wareing[10] and Nitsch[11]. They found with very different woody plants, that short days induce rest, while long

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days prolong growth. During early rest the effect of short days can be reversed by long light periods, but later on, when rest becomes deeper, it cannot be reversed any more by this treatment. At this stage chilling is required in order to break rest.

The perceptor of the photoperiodic stimulus is the leaf. As a matter of fact, we can prolong growth even into winter by defoliation. On the other hand in spring, towards the end of rest, deciduous trees will normally not have any leaves. Under particularly favourable conditions part of their leaves may however be retained. We had occasion to observe that, in these cases, the buds in the axil of such leaves will start to grow earlier than buds the subtending leaves of which have dropped. Thus, some substance or substances are formed in the leaves which affect the rest of the adjoining bud.

These phenomena are interpreted as due to a mechanism involving the interplay of auxins formed in light and inhibitors, some of which are destroyed by light, and most by cold. Before the application of paper chromatography to growth substances it was impossible to distinguish whether the disappaerance during rest of auxin activity was due to a decrease of 'free' auxin, as suggested by Bennett and Skoog[12] or, primarily, the difference between the activity of growth-promoting and inhibiting substances, as we suggested[13].

The first separations of these substances in woody plants were carried out by Hemberg[14] on *Fraxinus* and by Spiegel[15] in our laboratory on *Vitis*. They showed that the concentration of inhibitors clearly reflected the state of the rest period. Thus, in the grape vine (Fig. 1), the inhibitors reached a pronounced peak by the end of December, dropping to zero about two weeks before bud burst. They also disappeared after rest had been broken by cold treatment. Furthermore, comparing the curves for two *Vitis* crosses, *V. vinifera* × *V. rupestris* 1202, which requires little chilling, and *V. vinifera* × *V. berlandieri* 41-B, which requires severe chilling, we note that the general level of inhibitor concentration is proportional to the chilling requirement. Also, the inhibitor concentration in the *V. rupestris* cross reached zero

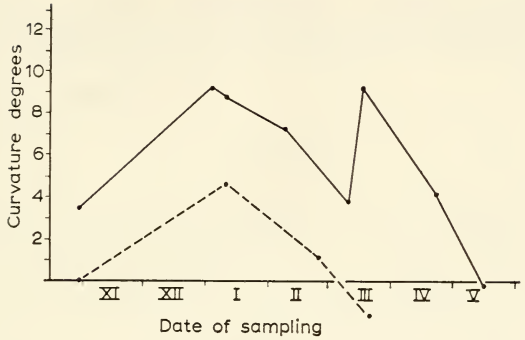


Fig. 1. Inhibitory activity in neutral fraction of ether extracts from grape buds of two *Vitis* crosses. Expressed as positive curvature of the coleoptile of *Avena* after Spiegel[15]. ———— = *V. vinifera* × *V. berlandieri;* — — — — — = *V. vinifera* × *V. rupestris*

in spring, about 70 days before the *V. berlandieri* cross. Since these investigations were published, similar results have been obtained with a number of woody plants, maximum inhibitor concentration always being found during 'mid rest'. On the other hand, with most plants growth-promoter activity is gradually reduced during entry into rest, disappearing entirely in some species during 'mid rest' and reappearing again towards the end of the rest period.

Considering growth-promoting substances, the activity of indoleacetic acid would seem to be very pronounced in most plants; indeed Spiegel considered it to be the only promoter in grape vine. In many plants indolethylacetate and indolepyruvic acid play an important part. Furthermore, the $R_F$ values of a few unknown growth-promoters have been reported. As regards inhibitors, two substances have lately been identified, but there would seem to exist quite a number of additional ones. In buds of peach trees Hendershott and Walker[16] identified naringenin (trihydroxyflavanone) and Housley and Taylor[17] revealed the mixture of substances making up Bennett–Clark's beta inhibitor,

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which seems to occur in many plants, as a number of aliphatic acids, unsaturated polyhydroxy fatty acids, azalaic acid and scopoletin.

As more of these substances and their enzymatic mechanisms become known, we will start to tread on more solid ground, whereas today we are dealing with uncertain hypotheses. Up to the present, research has dealt separately with different plant organs, presuming different mechanisms. Wareing and Black[18] have, however, pointed out that apparently the same mechanisms act in the different organs of the same plant—obviously there exist morphological modifying factors.

Permit me a concluding remark in connection with this particular gathering. I was very much impressed by the similarity of processes occurring in the diapause of insects and those which we find with buds of woody plants. Most of us working within relatively limited spheres of research know little about what is going on in disciplines as far removed as entomology from plant physiology. I think that this symposium will have made a considerable contribution by calling attention to those similarities.

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## DISCUSSION

Koller: You mentioned artificial wakening of buds as the result of an increase in acetaldehyde or anaerobiosis in buds, what happens in this respect as a result of the cold treatment?

Samish: It is thought that cold treatment destroys inhibitors.

Avi-Dor: Do dinitrophenol and related compounds convert the aerobic respiration to glycolysis? Usually dinitrophenol increases respiration. Might it not be a lifting of the Pasteur effect? Is there any proof for an increase in glycolysis?

Samish: This work has not been done with the type of material we are talking about, but it has been shown in animal tissues that energy-rich phosphorus bonds are not created and that respiration is directed into fermentative pathways.

Avi-Dor: The alternative explanation for the lack of respiration might be the absence of an ATP acceptor. The addition of dinitrophenol breaks down ATP and respiration can start. There must not necessarily be a conversion from aerobiosis to anaerobiosis.

Reinhold: I should like to point out in answer to Dr. Avi-Dor's earlier question that in both shoots and roots occurs a reversal of the Pasteur effect in the presence of dinitrophenol, with the occurrence both of anaerobic respiration and of increased glycolysis.

# ROUND TABLE DISCUSSION

MODERATOR: S. HESTRIN

The 'Round Table' lasted for two sessions and only a few comments can be cited here.

## SESSION I

Moderator (HESTRIN): We have been called to the 'Round Table' under an injunction to seek for simple formulations of the dormancy problem, and to try to find common denominators in the mechanisms of cryptobiosis as they occur in widely divergent forms of life. It will be one of our tasks, no doubt, merely to determine whether indeed it is reasonable at all to seek for such common denominators.

At first approach one is inclined to suspect that there may be at least two quite different classes of cryptobiosis which should be considered separately: (a) a transient class in which metabolic depression induced exogenously is promptly reversed when the environment is returned to normal; (b) a persistent class in which metabolic depression induced exogenously and/or endogenously, is not directly reversed on the reversion of the environment to normal.

### (*1*) *Anhydrobiosis*

Let us first consider a kind of cryptobiosis, which is perhaps of the exogenous kind—the state of anhydrobiosis induced by lyophilization. Would Dr. Kohn care to recapitulate for us some of his conclusions in this regard?

KOHN: In lyophilization you suspend metabolism by removing water. Most of my lecture dealt with the technical details of the method used in removing the water so as not to kill the organism in the process. This removal takes place in the frozen state, and one of our most important findings is that,

unless protected by special substances, the micro-organisms are killed in the dry state by oxygen. The hypothesis we proposed regarding the mode of the action of the oxygen invokes the paramagnetic properties of the molecule. Whether the hypothesis is valid in regard to micro-organisms other than those we have used, let alone other systems, remains to be learnt.

Moderator: Do you consider, Dr. Lees, that given the proper techniques all forms of life could remain viable when desiccated, as is the case with micro-organisms?

Lees: No, I think that most multicellular animals would succumb to complete desiccation. The explanation may have been provided by Prof. Halvorson when he emphasised the importance of structure in preserving viability. Desiccation leads to irreversible structural changes.

Moderator: Should we perhaps assume then that there has been in certain organisms a specific evolution of a mechanism which prevents structural injury on desiccation?

Lees: It would seem so. Although the extraordinary Chironomid *Polypedilum vanderplanki* obviously shrinks on desiccation, it must have some means of preserving essential structures from damage. In this respect it is almost unique: other species of bloodworms die as soon as they have lost about three quarters of their body water.

Halvorson: I would like to quote here the results of some experiments we performed in our laboratory some time ago. We measured the moisture affinity of some vegetative cells and in spores. This was done by measuring the equilibrium vapour pressure of the moisture in spores and in vegetative cells at different stages of drying. We found that in spores the vapour pressure remained close to that of pure water, until nearly all of the water had been removed, but in the vegetative cells the equilibrium vapour pressure fell rather rapidly while the moisture content was still relatively high. This would indicate that in the spore the water is free, that its removal does not affect the structure, and that there may be relatively little shrinking in a dry spore compared to a wet one. In vegetative

cells, however, the amount of shrinking on drying is of the same order as the shrinking in a drying piece of meat. This would explain the sensitivity of non-frozen vegetative cells to drying, and serves as still another example of the importance to be ascribed to structure conservation for the preservation of viability during drying.

### *(II) Resistance and Cryptobiosis*

Moderator: The endogenous class of cryptobiosis might perhaps be defined as a hypometabolic state whose sustained duration involves an endogenous regulatory mechanism. Would anybody like now to demolish this definition?

Koller: I have no intention of tearing it down without some previous study. But you should note that a state of dormancy is one that enables an organism to pass through an unfavourable environment without drying. It seems to me that the important criterion is the resistance to external conditions. It is this which determines whether a state is dormant or not.

Lees: I am afraid that this would not do for insects. If you take, say, resistance to cold, it is true that dormant insects are often resistant, but other insects, without a diapause, are sometimes equally cold-hardy.

Halvorson: As for micro-organisms, cells in a dormant state have an enhanced resistance to their environment. In bacterial spores we use loss of heat resistance as a means of determining whether dormancy has been broken.

Samish: In the cells of a bud going into rest the protoplasm becomes more viscous and less transparent whilst the proportion of bound water is increased. Bennett in Berkeley found a correlation between the depth of rest and the amount of bound water. With the breaking of rest the protoplasm becomes more liquid and the bound water percentage is decreased.

Halvorson: I was a member of the faculty of the University of Minnesota when Dr. Gortner first introduced the term: bound water. I then strongly disagreed with him, and I still think it is

a term that does not describe something that really exists. It can be found in the literature that the spore's resistance is increased because the water in the spore is bound. That is not so. Water in the spores is perfectly free. As I previously pointed out, one can evaporate almost all of the water of the spores and the vapour pressure will remain that of pure water.

LEES: I might perhaps add that certain species of insects are often found to be more cold-resistant in the dormant condition than when actively growing and developing. However, as R. W. Salt has shown, this may merely be a consequence of the presence of 'foreign' particles in the gut of the feeding insect. The physiological mechanisms responsible for dormancy and cold-hardiness may well be entirely different. We tend to think of insect diapause primarily as a growth phenomenon.

MANDELBAUM: We should also note that in trees frost-resistance and dormancy can be induced quite separately.

Moderator: Let us now ask the protozoologists and mycologists whether they think that resistance and metabolic dormancy are separable?

WAHL: Yes, indeed. In fungi they are entirely separable.

Moderator: We have decided, I think, that hypometabolic dormancy and enhanced resistance are separable phenomena though in some systems they are correlated.

MAYER: The correlation might arise as follows: a dormant organism has a lower requirement for energy-releasing processes. Therefore it is less affected by external factors. In the growing organism, on the other hand, there is a much higher requirement for energy-releasing processes. Hence external factors affect a growing cell more readily.

### *(III) Metabolism in Cryptobiosis*

Moderator: This brings us now to an important further question—whether the hypometabolic state is characterised by a qualitative change from normal in the metabolic pathways.

AVI-DOR: I would like to call your attention to the fact that

respiration in the hypometabolic state depends largely on systems which are insensitive to CO and narcotics. Such systems are not linked to the cytochromes, are not phosphorylating and perhaps do not yield much energy in a useful form. The assumption could thus be made perhaps that in the hypometabolic state the organism can afford to use a system which is energetically less effective but which is less sensitive to inhibition.

LEES: Investigators who have studied diapause in the *Cecropia* silkmoth have reached a different conclusion. Williams, Shappirio and Schneiderman are now of the opinion that the dormant state is not accompanied by any *qualitative* change in the metabolic pathway, only by an alteration in the *quantitative* relationships of the terminal enzyme, cytochrome oxidase, and cytochrome *c*. CN- and CO-resistance in the diapausing pupa is thought to be the result of the virtual disappearance of cytochrome *c* and the great excess of oxidase. This system, which would also be less effective energetically, would permit the stored reserves of fat and glycogen to be used sparingly.

HALVORSON: From what we know of the enzyme pattern of spores, it appears that while those enzymes needed for energy are preserved, a number of enzymes associated with biosynthetic reactions are acquired during outgrowth. Thus different enzyme patterns would be associated with the formation, maintenance and outgrowth from the dormant state.

LEES: Is it not possible that the maintenance respiration could be sustained by changes in the classical cytochrome system?

AVI-DOR: There is really no fundamental difference between the two views. The electron transport system might be the same up to a point, but if cytochrome *c* were missing, the final electron acceptor the reduced flavin proteins would have to be reoxidized directly by oxygen. Thus the pathway would still be largely the same but would break off at another point.

POLJAKOFF-MAYBER: Perhaps what is involved here is not so much a complete switchover as a change in proportion between the various pathways. I wonder whether there is not also a

structural aspect to be considered. The various components of a system can of course only interact if they concur in time and within a narrow space.

HALVORSON JR.: In answer to Dr. Poljakoff-Mayber, we have recently found mitochondrial type particles in vegetative cells of Bacillus. These particles were also present in the spores but they lacked a number of enzymes associated with cytochrome systems. The incomplete particles constitute a functional difference between spores and vegetative cells. I am rather inclined to believe that the differences between spores and sporulating cells must be very subtle, in view of the great similarity between a system going into the dormant state and the dormant state itself.

LEES: I think I mentioned in my lecture that mitochondria which are readily visible in the developing embryo of grasshoppers, 'disappear' when the embryo goes into diapause. They reappear when it comes out of diapause.

MAYER: May I enquire what exactly is meant here by disappearance and reappearance?

LEES: My remark was based on the work of Bucklin and his associates who have stained the mitochondria with Janus green B and examined the preparations with the light microscope. Actually, mitochondria are present during diapause but take the stain very lightly, indicating that there is a probable deficiency in the cytochrome system.

HALVORSON JR.: In spores one observes under the electron microscope particles very similar to those in the vegetative cell. The mitochondria-like particle in the spore is non-functional. It contains several of the enzymes but does not contain a complete oxygen transport system. In the vegetative cell there is a small quantity of the soluble DPNH oxidase. In the spore its quantity is very high and accounts for all of the passage to molecular oxygen. So you employ alternate systems as you go from the spore to the vegetative cell, or the other way.

MAYER: In seeds, too, one suspects such a switch in electron transport systems with dormancy. It is very pleasing to hear of an even clearer case of it in the bacterial spore.

KEYNAN: Does not a change of this sort in fact also occur in insects?

MAYER: From Dr. Lees' comments it seems that as far as insects are concerned the verdict should be a 'not proven' rather than a 'nonexistant'. It seems to me that by the use of the Britton-Chance spectrophotometer technique we could learn whether or not there is a change in cytochrome $b_5$ and cytochrome $c$ in diapause, and thus show whether or not the cytochrome system is operative in this stage.

LEES: A dramatic fall in cytochrome $c$ concentration has in fact been demonstrated by Shappirio using low temperature spectroscopy.

POLJAKOFF-MAYBER: We cannot be sure that there is no previously accumulated energy-store in the form of ATP. This store could be used during dormancy. After all, in the latent state of metabolism there may not be much need for energy.

HALVORSON JR.: We do not know the ATP/ADP ratios in the spore. It would be worthwhile indeed to measure this. There are only FitzJames' observations on this subject, on a correlation between germination and polyphosphate degradation.

MAYER: The polyphosphate, phytin, might be such an energy store in a dormant seed.

Moderator: The difficulties of the approach to this problem are formidable. I would not wish to sound pessimistic, but please realize that whereas in the bacterial system we know what cell is dormant, and can test that cell, in insects and plants there appears to be as yet no accurate location of dormancy, and no means therefore of differentially examining the critical locus.

SAMISH: Well, in buds, some think that the scales form a wall which prevents the entry of oxygen and thus induces dormancy, but others hold the opposite view, that it is the dormancy which induces the formation of an impermeable protoplasmic membrane. A group of Russian workers has correlated entry into dormancy with constriction of the protoplast and resultant scission of the plasmodesma. They have also found that when

there is less resistance to cold there is less tendency to the breaking of the plasmodesma.

LEES: It depends on what you regard as the critical locus. The organ systems controlling diapause in insect eggs, larvae and pupae are different. However, although diapause mechanisms, even in closely related species, have probably been evolved independently, natural selection has had to act on existing growth-controlling systems, such as the endocrine system. Therefore a certain uniformity emerges when the mechanisms of particular stages are compared. Some further similarities, especially in connection with respiratory metabolism, appear if the reacting tissues are regarded as the critical locus.

HALVORSON: In micro-organisms, those most resistant to the environment are spherical. Spores, too are all more or less spherical. I do not know though whether this is in any way significant.

Moderator: It might be rewarding to focus our discussion upon the surface of the dormant organism. Is it not true that such a surface is generally waxy or at any rate hydrophobic?

LEES: Well, as most insects possess a waterproof cuticle throughout development one would have to find out whether the waterproofing was more efficient during diapause. I do not think that this has yet been studied critically. It is perhaps worth mentioning, however, that silkworm pupae show a visible accumulation of wax after they have remained dormant for a year or two.

WAHL: A morphological feature common to many fungi in the dormant stage is the thick wall which reduces penetration.

HALVORSON: It is true that spore cell walls are different from the vegetative cell walls. It is also probably true that Gram positive organisms, which are more resistant have a cell wall differing from that of the less resistant organisms. There may be a correlation between the type and composition of the cell wall and the dormancy and resistance of the cell.

*(IV) Induction of Cryptobiosis*

Moderator: Perhaps if we make a list of elements which induce or break dormancy in different organisms we might find a few common denominators for the different systems.

HALVORSON: Dormancy in micro-organisms was once thought to be induced by unfavourable conditions. This is not so. But I think it is true that the inducement to form spores is occasioned by a change in the nutritional environment, and this perhaps may be true in other systems as well.

KOLLER: The basic similarity in the various dormancy-inducing and -breaking mechanisms is the fact that they are all triggered by the environment.

LEES: I agree. We have factors like dryness and lack of food as well as guide factors such as photoperiod. The response to the latter allows anticipation of unfavourable conditions. The dormancy-controlling factors are remarkably similar in buds and insects. In both cases induction is by photoperiod and breaking by chilling. But how far does this similarity really go? The guide stimuli are shared, but different response mechanisms have been evolved.

Moderator: It is a credo of biochemists that there must be some common mechanism. We can only discover it by looking 'under the skin'. The zoologist is trained to see many differences and dissimilarities. Specifically one asks whether the several photoreceptive and 'endogenous clock' mechanisms encountered in different species are not in principle similar? We know so little, it is perhaps better not to try to answer this particular question yet. We are now concluding our discussion for today, but I would very much like you to give some thought to the following questions by way of preparation for our second session. Is there perhaps an important experiment which you would like to see performed in this field? If so, would you like to describe it? Secondly, could you point to a biological system particularly suitable to the study of cryptobiosis? Can you suggest quantitative procedures which would facilitate a more exact approach to our problems?

Finally, would you care to say whether cryptobiotic states are a useful feature in evolution?

SESSION II

## *(V) The Selection of Suitable Biological Materials*

Moderator (HESTRIN): A proper choice of biological material for the examination of the problems of dormancy could no doubt be a matter of critical importance for the advancement of knowledge in this area. Could we perhaps arrive at some agreement as to the kind of properties we would want in such biological objects of our choice? Perhaps Prof. Halvorson who has done so much of the pioneer work on this problem would consent to give us his opinion as to the manner in which the choice objects for studies should now be made?

HALVORSON: Little progress was made in the study of the physiology of the bacterial spore until it was realized that proper separation of the dormant stage (ordinary spore) was essential. I am sure this is applicable to all the other fields.

Moderator: I suppose heterogeneity in the bacterial spore population might be a serious obstacle to quantitative studies of the phenomena. Is it now possible to get synchronized germination in homogeneous spore samples?

HALVORSON JR.: I think that variations in time of germination result from variations in time of storage of the spores. As activated forms of spores show considerable loss of weight, we have been able to perform separation by equilibrium sedimentation in the density gradient. Another heterogeneity was in resistance to ethylene oxide resulting from phenotypic variation in fat content against a constant genetic background. Here we accomplished a separation by electrophoresis. Thus by use of appropriate physical techniques one can get greater population homogeneity.

KOHN: Would density gradient centrifugation ensure homogeneity in spores?

HALVORSON JR.: We know we can separate active spores from

dormant ones by this method, and also heat-shocked spores from dormant spores. By refining the density differences, one should be able to separate spores with varying degrees of dormancy.

Moderator: As a bystander one has the impression that microbiologists are fully aware of this aspect of the problem and that they are directing adequate efforts to the working up of reliable assay procedures. This may not be equally true, however, in relation to some of the more complicated biological systems. Would you like to comment, Dr. Lees, on good quantitative techniques for work with insects.

Lees: The selection of the most suitable and the most standardized material for insect dormancy studies has not really been a problem. You choose your insect according to your purpose; for endocrine system studies you choose a large insect, like a giant silkmoth. For studies on, say, photoperiod, a quickly multiplying species is preferable. Unfortunately, such an insect is usually an inconveniently small subject for surgery. *Lepidoptera* of moderate size have nevertheless been used rather extensively for both purposes. The silkworm of commerce, with its different genetic races, has been a particularly favoured species.

Moderator: Might it not be useful to concentrate within a given insect on one particularly suitable tissue?

Lees: If you mean a tissue suitable both for biochemical work and for the study of cell structure during dormancy, the epidermis should prove to be a good choice. It forms a flat layer only one cell thick and is easily stained and preserved as a whole mount. Wigglesworth has shown that when moulting is induced by injection of ecdyson, detectable cytological and cytochemical changes occur within 24 h. Shappirio has also used wing epithelium for *in vivo* spectroscopic studies.

Moderator: Could one cut this tissue up and put it in a test tube?

Lees: The epidermis could certainly be separated and studied biochemically. But if you are referring to the possibility of tissue culture, the prospects are not bright. As for organ culture,

media are known in which lengthy survival is possible, but there is usually no growth.

HARPAZ: We were recently contemplating an extraction of haemolymph from insects with a view of separating the haemolymph cells and growing them in culture. Should this succeed we might also have a suitable material for dormancy studies. Some of these cells are fairly large and can be studied with the ordinary light microscope. We could get about a couple of milliliters of material from a fairly large insect.

KINDLER: Bearing in mind Prof. Shulov's suggestion that lack of movement is a sign of dormancy in insects, might it not be a good idea to use muscle tissue?

LEES: There would be no technical difficulty. You could dissect the muscle tissue free from anything else, except hemocytes from the blood. However, the muscle tissue of an insect in diapause may not show any characteristics peculiar to the diapause condition. In the *Cecropia* silkworm, the muscles contain their full complement of enzymes and are still fully functional; thus the insect is able to wriggle its abdomen.

GALUN: Even if we did succeed in getting a tissue culture, it would be no benefit for the study of this aspect of dormancy. The concept of diapause does not apply to the cells once they have been removed from the regulatory system of the body.

Moderator: Perhaps we should not take so extreme a view. It should be remembered that in the mammalian system it was for a long time generally thought that no hormone effect could operate outside the intact organism. Yet this is no longer so.

GALUN: No, I assume some hormone effect could be obtained, but not in the case of arrested growth.

Moderator: What is the position in this regard as to fungi?

WAHL: I am afraid that it will be extremely difficult to find among fungi genetically pure and uniform material for dormancy studies.

NACHMONY: A liverwort which I have been working with might be a particularly suitable test object. In this organism, one

can induce dormancy by 16 long days, and break it by 3 short days. All the stages of the life cycle manifest this response pattern. I tried it out both on the whole gametophyte and on the thallus which I had cut into pieces, and obtained the same results in both. Another advantage of this material is that the gemmae—the vegetative reproductive organs are genetically uniform. Dormancy can be induced in them in the gemmacurp. One can thus obtain at will either dormant or nondormant gemmae. We have so far observed these phenomena but know very little about the biochemical behaviour. The suitable temperature for growing this material is about 15° and the required light intensity about 120 ft.-c.

Moderator: This seems to be truly a most attractive and uniquely suitable biological material for the study of dormancy.

### *(VI) Memory Phenomena in Dormancy*

Moderator: Several of the speakers have pointed to the operation of the memory phenomena in relation to induction and duration of dormancy in different systems. What might be the physical basis of these effects?

Lees: The mechanism controlling diapause termination provides a kind of memory, usually of past temperatures which are integrated in a very particular way. As a model system we could suppose that two chemical reactions are competing for a given substrate; or, simply, that there are opposing synthetic and breakdown reactions. In both cases the reaction must have different temperature coefficients. As yet we have no inkling as to what these hypothetical reactions might be.

The neurosecretory cells are involved in another type of memory when, in diapause induction, they 'record' the photoperiodic stimulus even though its effect is not manifested until much later in development. The photoperiodic measurement of time itself involves a third kind of memory, the nature of which is still largely obscure.

*(VII) Proposals for Future Work*

Moderator: Let us consider finally experiments still to be performed on the induction and the breaking of dormancy in the different biological classes.

HALVORSON JR.: A simple explanation for many dormancies is that they are simply expressions of an anhydrous condition. A way of investigating this might be the exposure of a system to deuteriated or tritiated water. Then after rupturing the tissue in a non-aqueous solvent, we could look for deuterium or tritium on some recognisable internal component. One would then find whether water could enter such a component. The other question is whether there is any metabolism in the anhydrous system. We might be able to decide this point by determining whether there is incorporation of $^{32}P$ into ATP within the 'anhydrous' cell.

JASHPHE: It might be very important to determine whether variations in the permeation barrier accompany the induction and breaking of dormancy.

HALVORSON: I agree this is very important, but the design of a suitable experiment on this is a very difficult problem.

MAYER: In bacterial spores, it is very often a heat shock that breaks dormancy. In seeds too, a heat shock of short duration and at moderately high temperature performs the same function. All this seems to point to some change in the physical structure. However, I have no idea how to establish what this structure is.

WAHL: I have two suggestions. One is based on the observation that when we inoculate oat seedlings in one-leaf stage with uredospores of race 2 of *Puccinia graminis avenae*, uredia are produced on the leaf and telia are formed on the coleoptyle. In other words, the same inoculum produced the active stage of the rust fungus on the leaf and the dormant stage on the coleoptyle. The question arises why does a similar genetic stock yield on the same plant dormant and non-dormant spores, depending only on the kind of tissue used as a host. The second proposal stems from Yarwood's studies on the powdery mildew of red clover, *Erysiphe polygoni*. Conidiospores collected during

the day germinate readily while those collected at night germinate poorly or not at all.

The mechanism underlying these phenomena deserves further elucidation.

HALVORSON: I have no pet experiment to suggest, but I do want to make a general suggestion, applicable perhaps to all the fields. It is generally agreed that the study of bacterial spores has arrived at a rather ideal technique. The reason for this is, that the investigations were centered for a long time on the initial changes rather than on the phase of outgrowth. In doing so only germination triggering was studied. Therefore some similar techniques would have to be found in the other fields before some real progress could be registered.

### *(VIII) Purpose of Dormancy*

Moderator: There is one further aspect we can still touch on briefly: the purpose of dormancy. This is of course teleological, but I think this is permissible on the last day of the conference. Do we have dormant stages which are useful as distinct from resistant stages?

HALVORSON JR.: I would like to quote some work done by Dr. Knight and his colleagues at Wisconsin University on the enzymatic oxidation of sterols. They found that this reaction takes place only in the spore and is absent in vegetative cells. The presence of specific antigens in bacterial spores, of cysteine-rich structures as shown by Vinter, of heat-resistant enzymes and of compounds such as dipicolinic acid, all attest to distinctive features of the dormant state in bacterial endospores.

LEES: I accept the invitation to become teleological! I have emphasized that the diapause state does not necessarily go hand in hand with resistance to adverse environmental factors such as low temperature and water lack. Diapause should rather be regarded as a timing device which ensures that the actively developing feeding stages appear at a time when these factors are favourable. When this delicate adjustment is upset by

removing the insect from its natural environment, dramatic consequences may follow. I have been told that the Cynthia silkmoth was once quite common in New York where it produced two broods a year, as it did in its native South East Asia. In New York, however, average temperatures are often higher and in one particularly hot summer, a third generation was begun which failed to reach completion before the *Ailanthus* trees lost their leaves in the fall. The resulting population crash has remained a permanency.

Moderator: We have now come to the end of our discussion. I would like to thank all of you for your active participation. I am wondering what the position will be in a few years when we meet again to discuss cryptobiosis. I believe the whole classification of the subject will be different and perhaps Dr. Keynan might consider the reconvening of this conference in a few years.

On behalf of all of you, I would like to thank him for the hospitality he has extended to us. I would also like to thank most warmly our guests the Halvorsons—father and son, and Dr. Lees for their most stimulating contributions to our discussions, and finally all of you for the friendly and scientific spirit in which our work was conducted.

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